THE FATE OF ENDOZOOCHOROUSLY DISPERSED SEEDS

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The fate of endozoochorously dispersed seeds

Het lot van endozoöchoor verbreide zaden

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Tanja

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1 General introduction

Sheep grazing in the nature reserve 'De Zoom-Kalmthoutse heide', Kalmthout, Belgium (autumn 2016). ©Bastiaan Notebaert

1.1 Introduction

1.1.1 Seed dispersal

Dispersal of plants

Dispersal can be defined as the tendency of an organism to reproduce away from its place of birth (Duputié and Massol, 2013; Levin et al., 2003). It hence determines the outcome of many evolutionary and ecological processes such as speciation and extinction events and the coexistence of species and genotypes within species. In the case of sessile organisms such as plants, dispersal is largely limited to the last steps in the life cycle. In vascular plants dispersal is often closely linked to sexual reproduction as the most prevailing dispersal units are seeds and fruits, although vegetative structures such as rhizomes, bulbils or even the entire plant can be adequate dispersal propagules as well (Poschlod et al., 2005). Both vegetative and reproductive dispersal units are often united in the term *'diaspores'* which stems from the Ancient Greek word *'diasporá'* (dispersion) or from '*diaspeiro*' (I scatter).

Dispersal of plants can be seen both on a spatial and temporal scale. Dispersal in space involves the movement of dispersal units over certain distances whereas dispersal in time or dormancy postpones germination which enables the incorporation of seeds into the soil seed bank even if the environment is suitable for germination.

In this work, I will focus on several aspects of the dispersal of sexual structures of vascular plants on a spatial scale. Therefore, the broad term of *'seed dispersal'* is used throughout this thesis as a general expression of the movement of the reproductive units of plants although for some species the fruits or various combinations of fruits and other plant parts (e.g., the achenes and mericarps of Cyperaceae, Rubiaceae and Urticaceae species, or the diaspores of Poaceae species which consist of a caryopsis, lemma and palea) are the actual dispersal units.

Implications of seed dispersal

Seed dispersal, being the mobile link between the end of the reproductive cycle of the adult plant and the start of a new one by the establishment of its offspring, has a profound effect on vegetation structure and dynamics (Wang and Smith, 2002). The subsequent steps of seedling emergence, establishment and reproduction are crucial processes in plant population dynamics and the spatial structure of plant communities as they shape the distribution and abundance of plant species (Howe and Smallwood, 1982; Wang and Smith, 2002; Wenny, 2001). Three mutually not exclusive hypotheses have been proposed for explaining the advantageous effects of seed dispersal for plants. Firstly, according to the Janzen-Connell hypothesis seed dispersal helps seeds and seedlings to escape from high mortality rates near the parent plant caused by density-dependent factors such as pathogens, seed predators, and/or herbivores (*'escape hypothesis'*) (Connell, 1971; Janzen, 1970). This implies that those seedlings that are growing furthest from the parent plant are given a survival advantage by avoiding inbreeding and intraspecific competition with other seedlings and the mother plant. The second hypothesis states that dispersal potentially brings seeds to rare and unpredictable sites that are fit for the establishment of new populations (*'colonization hypothesis'*) (Cain et al., 2000; Howe and Smallwood, 1982). The colonization model is most relevant when the targeted colonization sites are unpredictable and randomly distributed. One possible strategy for maximizing the chances of reaching those sites which are currently suitable for colonization or which will become suitable in the future would be by dispersing seeds in a wide area around the parent plant (Wenny, 2001). A third hypothesis states that in some cases disproportionate amounts of seeds are dispersed towards micro sites characterized by the specific and narrow range of environmental conditions favouring establishment (*'directed dispersal hypothesis'*) (Howe and Smallwood, 1982; Wenny, 2001). The theory of directed dispersal is based on the non-random distribution of appropriate deposition sites and the survival of seeds in predictable environments (Wenny, 2001). As certain dispersal mechanisms are more likely to transport seeds to suitable habitat patches, strategies favouring directed dispersal often adapt the morphology of seeds or fruits in a way that one dispersal mechanism is favoured over another (Venable and Brown, 1993). Although these three hypotheses are often put forward in explaining the advantages of seed dispersal for plant

populations, measuring or even distinguishing each of these strategies is not easy. The three identified advantages of seed dispersal are not mutually exclusive as many plants benefit from more than one advantage of seed dispersal (Wenny, 2001). For example, it is perfectly possible that seeds get dispersed non-randomly to suitable sites (supporting the directed dispersal hypothesis) while escaping from high mortality near the parent plant (supporting the escape hypothesis). Also, most seed dispersal does not result in seedling establishment which implies that dispersal patterns are difficult to find, especially on large spatial scales.

Another factor to consider when evaluating the relevance of seed dispersal in plant ecology is the distance over which seeds get dispersed. Seed dispersal curves are typically leptokurtic which implies that the vast majority of seeds travel short distances and get deposited near the parent plant (Poschlod et al., 2005). Nonetheless, the few seeds that manage to travel over long distances are more interesting in vegetation ecology. Long-distance seed dispersal enables plants to colonize new habitats or to connect different populations (e.g., the colonization of islands, response to global change and post-glacial migrations). It is therefore one of the key aspects in metapopulation dynamics and in vegetation dynamics and diversity, but it also facilitates the spread of invasive species (Cain et al., 2000).

Mechanisms of seed dispersal

Seed dispersal mechanisms are often classified according to the dispersal agents or dispersal vectors. Dispersal vectors are either abiotic (wind, water or gravity) or biotic (animals including man, or the plant itself) and are related to diaspore morphology (Albert et al., 2015b; Levin et al., 2003). While the morphology of diaspores often is interpreted as an adaptation to a specific dispersal vector, dispersal units can in theory be dispersed by every vector over a wide range of distances (Poschlod et al., 2005). The ability to be dispersed by multiple dispersal vectors is a common phenomenon in terrestrial ecosystems. In a meta-analysis of the dispersal traits of the Dutch flora and the position of plant communities along major environmental gradients, Ozinga et al. (2004) found on average 2.15 dispersal vectors per species (out of 5 options: wind, water, the fur of large

herbivores, the dung of large herbivores, and birds). Furthermore, the mean number of potential long-distance dispersal vectors increases with environmental factors such as light availability (Ozinga et al., 2004) while the probability of dispersal by one of the different dispersal vectors and the distance potentially covered varies greatly, and is closely linked to diaspore morphology (Albert et al., 2015b). A wide range of morphological adaptations to promote the probability and distance of dispersal exists, e.g., the achenes of dandelions or the dust-like seeds of orchids promoting dispersal by wind ('*anemochory*', e.g., Andersen (1993), Eriksson and Kainulainen (2011)), the dispersal of buoyant seeds and plant parts of marine macrophytes by water ('*hydrochory*', e.g., Harwell and Roth (2002)) and the prevalence of explosive fruits ('*autochory*' or more specifically '*ballistochory*' e.g., Stamp and Lucas (1983)). Animal dispersed seeds often have adaptations to attract their dispersers, such as highly nutritive fleshy fruits attracting frugivorous birds, mammals (e.g., bats, primates, elephants and foxes), and reptiles ('*endozoochory*' or more specifically in case of fleshy fruits *'frugivory'*, see e.g., Snow (1981), Fleming and Heithaus (1981), Dew and Wright (1998), Campos-Arceiz and Blake (2011), Traba et al. (2006), Olesen and Valido (2003)). In most cases of animal-assisted seed dispersal, plants try to attract a wide range of seed dispersing herbivores, while others invest in specialized structures and/or chemical compounds to attract one specific type of dispersal vector. One remarkable example of selecting dispersal vectors has been found in the directed deterrence strategy of chillies (*Capsicum*) (Tewksbury and Nabhan, 2001). The presence of capsaicin, which is responsible for the peppery heat of the fruit, discourages mammals from consuming the fruits while birds, being the most effective dispersers for this plant species, are not deterred. Another example of chemical attractants are the elaiosomes found in certain *Corydalis*, *Lamium* and *Viola* species. These lipid filled structures are attached to the seed coat and contain chemical compounds that attract ants. The seeds are consequently carried back to the nests by ants where the elaiosomes are eaten and seeds are discarded intact ('*myrmecochory*', e.g., Brew et al. (1989), Hughes and Westoby (1992)). Other plant species invest in appendages such as hooks or bristles, or cover seeds or fruits by a viscous layer to enhance the chance of getting attached to the external body parts of animals ('*epizoochory*', e.g., Couvreur (2004b), Vivian-Smith and Stiles (1994)). In contrast, many herbaceous species lacking any apparent morphological adaptations to any dispersal mode are often found

germinating in herbivore dung which is especially the case in temperate regions (Cosyns et al., 2005a; Mouissie et al., 2005c; Pakeman et al., 1998; Willson et al., 1990). The *'foliage is the fruit'* hypothesis postulates that grazing herbivores are attracted to the palatable foliage of dry fruited herbaceous species and ingest the small and inconspicuous seeds rather inadvertently (Janzen, 1984).

Seed dispersal often is a complex process containing multiple steps with different dispersal vectors (*'diplochory'*, see Vander Wall and Longland (2004)). In most cases, diplochory contains two dispersal phases by different vectors, but three or more phases are possible as well (Vander Wall and Longland, 2004). While the first phase of dispersal moves seeds away from the parent plant reducing competition between seedlings and the parent plant and enabling the colonization of new habitats, the second phase of dispersal often directs seeds toward safe sites (e.g., underground) (Figure 1.1). Combining multiple dispersal mechanisms often provides greater benefits to seeds than each dispersal mechanism alone (Chambers and MacMahon, 1994; Vander Wall and Longland, 2004). Many examples of diplochory have been identified, such as the combination of autochory and dispersal by ants in *Viola* species containing an elaiosome (e.g., Beattie and Lyons (1975)), ballistic dispersal and rain wash (e.g., Stamp and Lucas (1983)), wind dispersal and scatter-hoarding by secondary dispersers such as chipmunks, jays and mice (e.g., Thayer and Vander Wall (2005), Beck and Vander Wall (2010)), endozoochory and scatter-hoarding rodents (e.g., Janzen (1982b)), frugivory and myrmecochory (e.g., Passos and Oliveira (2002)), endozoochory combined with the removal of seed-containing dung by dung beetles (e.g., Andresen (2002a), Beaune et al. (2012), D'hondt et al. (2008)), and the predation of frugivorous birds or lizards by birds which is believed to be an important long-distance dispersal mechanisms in island archipelagos (e.g., frugivorous lizards and kestrels in the Canary islands (Nogales et al., 2012; Nogales et al., 2007; Padilla and Nogales, 2009), and frugivorous finches and owls on the Galápagos (Grant et al., 1975)).

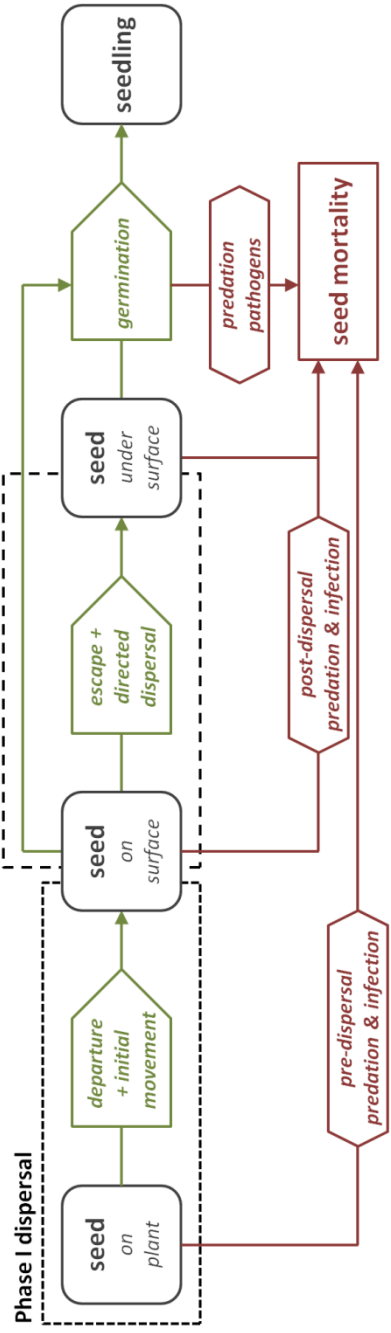


Figure 1.1 - Possible pathways in diplochory. In green, the main advantages of seed dispersal are given for each step in the dispersal process while in red the pathways leading to seed mortality are shown (modified from Vander Wall and Longland (2004)).

Endozoochory and the post-dispersal fate of seeds

The internal transport of seeds has been reported for many taxonomic groups, including frugivorous birds (e.g., Wenny (2000)), beetles (de Vega et al., 2011), rabbits (e.g., Malo et al. (1995)), foxes (D'hondt et al., 2011), domestic herbivores (e.g., Cosyns et al. (2005a), Eichberg et al. (2007)), up to the largest terrestrial herbivores (Campos-Arceiz and Blake, 2011). Despite this wide array of endozoochorous dispersal vectors, the availability and efficiency of individual dispersal vectors differ according to environmental factors and seed traits (Albert et al., 2015a; Albert et al., 2015b; Ozinga et al., 2004). Endozoochory by large grazing herbivores could be one of the main dispersal mechanisms of long distance dispersal due to the combination of large home ranges, high travel velocity, large gut size and long seed retention times (Cousens et al., 2010; Nathan et al., 2008; Pakeman, 2001). Furthermore, many germinable grassland seeds have been found highly concentrated in herbivore dung patches which suggest that endozoochory is one of the main drivers shaping temperate grassland communities (Cosyns et al., 2005a; Couvreur et al., 2005a; Pakeman et al., 2002).

Several steps in endozoochorous dispersal can be identified (Figure 1.2), and endozoochorous dispersal can only be considered successful if certain conditions are met. First of all, herbivores should ingest diaspores, which mainly depends on plant palatability and nutrient content (Mouissie et al., 2005c), seed production (Malo and Suárez, 1995b) and plant cover (Cosyns et al., 2005a). Secondly, seeds should survive their journey through the digestive tract. Seed feeding experiments have resulted in contrasting outcomes with increased germination in some cases (e.g., Manzano et al. (2005), Ramos et al. (2006), D'hondt and Hoffmann (2011), Mancilla-Leyton et al. (2011), Grande et al. (2013)) and reduced germination in others (e.g., Cosyns et al. (2005b), Manzano et al. (2005), Mouissie et al. (2005b), D'hondt et al. (2011), Grande et al. (2013)). Successful endozoochorous dispersal therefore involves the resistance of seeds during the consecutive digestive processes (mastication, rumination and digestion), and the resulting chemical, thermal and mechanical processes may result in the breaking of dormancy and thus in enhancing or reducing germination success and timing (D'hondt and Hoffmann, 2011; Traveset, 1998). Determinants of effective seed transfer through the gastrointestinal tracts

of herbivores are related to plant traits such as diaspore size and shape, permeability of the seed coat, seed longevity and seed production (Bruun and Poschlod, 2006; Cosyns and Hoffmann, 2005; Mouissie et al., 2005b; Pakeman et al., 2002), and herbivore traits such as diet, digestive system, seed retention time and body mass (Clauss et al., 2003; Cosyns and Hoffmann, 2005; Van Weyenberg et al., 2006). Thirdly, seeds should be able to germinate and grow to adult plants in a dung environment. While the first two prerequisites of endozoochory have gained much scientific attention in recent decades, and, therefore, provide valid proof for the existence and relevance of this dispersal mode, the next steps in plant establishment are far less studied. Nonetheless, the decisive step in seed dispersal depends on the post-dispersal fate of seeds and includes the processes of secondary seed dispersal, seed predation, germination, establishment, growth and the outcome of competitive processes (Figure 1.2, Traveset et al. (2001), Nathan and Muller-landau (2000), Wang and Smith (2002)). The deposition of viable seeds in a highly nutritive, though largely organic environment may affect germination and the establishment of seedlings (Deshaies et al., 2009; Greipsson and Davy, 1997), biomass (Deshaies et al., 2009), seed production (Greipsson and Davy, 1997) and phenology (Nomura and Kikuzawa, 2003; Nord et al., 2011; Power et al., 2006). Besides the high concentration of growth promoting macronutrients (N, K and to a lower extent P, Ca and Mg) and essential trace minerals (e.g., Fe, Mn and Cu) (Haynes and Williams, 1993; Lupwayi et al., 2000), animal excretions also contain growth inhibiting substances such as phenolic compounds and fatty acids (Marambe et al., 1993). As these phytotoxic compounds may alter the activity of enzymes that regulate the germination rate, the effect of dung on the germination and establishment of seedlings is a complex matter. Furthermore, competitive processes between seedlings within the dung pat and with the surrounding vegetation may further shape the outcome of the dispersal process. Large excrements often suppress the existing vegetation and create, accordingly, suitable microhabitats for germination and establishment by reducing competition with the already developed vegetation (Traveset, 1998). On the other hand, dung pats often contain a high concentration of germinable seeds belonging to a wide range of species. Although seed density varies with seed availability in the environment, herbivore type and size, and season (Cosyns et al., 2005a; Malo and Suárez, 1995b), the high variability of seed concentrations and species combinations found in the rather limited surface area occupied by

dung pats suggests that interspecific and intraspecific competition has a considerable impact on post-dispersal seed fate.

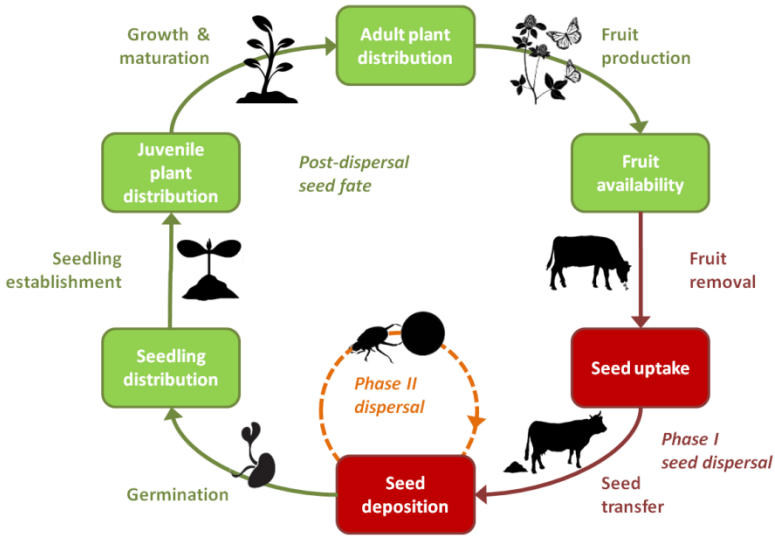


Figure 1.2 - The seed dispersal cycle modified from Wang and Smith (2002). Processes marked in red represent the consecutive steps of seed dispersal *sensu stricto* while processes marked in green determine the post-dispersal fate of seeds. Optionally secondary seed dispersal may take place.

Although primary seed dispersal by large herbivores enables seeds to escape the high seedling mortality near the parent plant (Connell, 1971; Janzen, 1970), seeds are still deposited in high concentrations in dung. Therefore, further dispersal events such as secondary seed dispersal (Vander Wall and Longland, 2004) and post-dispersal seed predation (Manzano et al., 2010) could lower the competitive pressure for the seeds remaining in the dung pat. Dung beetles often act as secondary dispersers of the seeds already present in dung (Andresen and Feer, 2005; Vander Wall et al., 2005). As dung beetles are able to locate fresh dung deposits very quickly, transport it over short distances and often bury it shallowly, secondary dispersal by dung beetles could largely account for the patterning of plants in communities and ecosystems (Chambers and MacMahon, 1994; Estrada and Coates-Estrada, 1991). Dung beetles mainly use herbivore and omnivore

dung as a food source in the adult phase and for nesting (Barbero et al., 1999; Finn and Giller, 2002; Gittings and Giller, 1998) and generally do not treat dung containing seeds differently than seed-free dung, given that the seeds are relatively small in comparison to the beetle (Andresen, 2002a; Braga et al., 2013; Slade et al., 2007). As dung beetles are a globally distributed insect group (Cambefort, 1991), and given the globally observed dung redistribution patterns caused by dung beetle activity, it can be expected that they fulfil similar functions in diverse terrestrial ecosystems throughout the world (Nichols et al., 2008).

Costs and benefits of endozoochorous seed dispersal

Often two types of costs are assigned to endozoochorous dispersal which include the investment of the plant in producing palatable fruits and seeds suited for surviving the intestinal tract (*energetic costs*), and the mortality of seeds during or after the dispersal event (*risk costs*) (Venable and Brown, 1993). A third cost could encompass a slower growth or a reduced reproductive output of the next generation (*opportunity cost*) (Bonte et al., 2012). While the first mentioned investment is applicable for both dispersed and undispersed seeds, the latter three costs are directly related to the dispersal event itself and can be considered as 'direct' dispersal costs. Benefits of endozoochorous dispersal are related to the possibility of long distance dispersal enabling the colonization of new habitats and the genetic exchange between meta-populations (Bohrer et al., 2005; Cain et al., 2000; Levin et al., 2003), and with the before-mentioned advantages of escaping the direct vicinity of the parent plant (Connell, 1971; Janzen, 1970), the directedness of dispersal to suitable habitats (Howe and Smallwood, 1982; Wenny, 2001). As grazing herbivores prefer certain habitats over others (e.g., Lamoot et al. (2005b)) and defecate while grazing (Lamoot et al., 2004), the probability that endozoochorously dispersed seeds are deposited in a similar habitat type is higher than expected from a random distribution (D'hondt et al., 2012). Furthermore, seeds of certain species germinate better after passage through the gastrointestinal tract (e.g., D'hondt and Hoffmann (2011)), and the specific environment of dung could result in enhanced growth and reproductive output (Bakker and Olff, 2003; Cosyns et al., 2006).

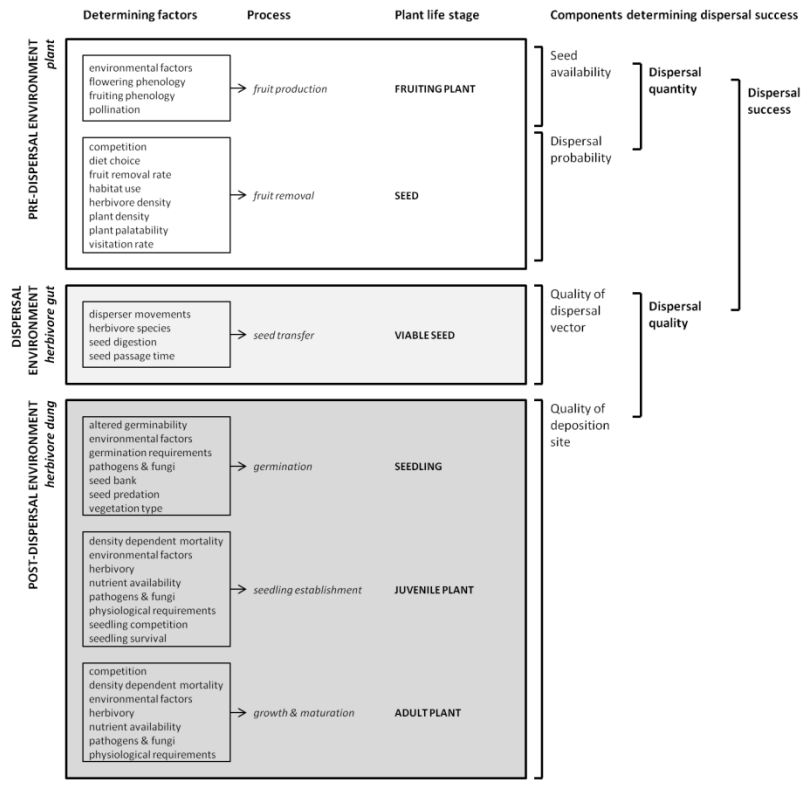


Figure 1.3 - Scheme summarizing the determinants of the outcome of the seed dispersal process. Factors determining the consecutive processes in endozoochorous seed dispersal are listed and the corresponding plant life stages (modified from Wang and Smith (2002) and Schupp et al. (2010)).

The notion that endozoochorous seed dispersers differ in effectiveness (Wheelwright and Orians, 1982) has led to the development of the seed dispersal effectiveness (SDE) concept by Schupp (1993). In the SDE framework, effectiveness not only includes the dispersal of seeds as such, but also the successful establishment of new individuals (Schupp et al., 2010), and is a function of the quantity of the seeds dispersed and the quality of the dispersal process and deposition (Herrera and Jordano, 1981; Schupp, 1993). The number of seeds dispersed can be estimated from the production of seeds on the parent plant and the probability that seeds or fruits are ingested by the herbivore and can be merely situated in the pre-

dispersal phase of flowering and fruit production (Figure 1.3). The effects of the quality of dispersal can be assigned to the dispersal and post-dispersal phase as both the quality of the disperser (e.g., gastrointestinal environment, disperser movements) and the quality of the deposition site determine whether viable seeds end up in a location suitable for the establishment of adult plants.

Fresh dung pat of American bison in a snowy landscape at Yellowstone National Park, USA (November 2015). ©Bastiaan Notebaert

1.1.2 The role of dung beetles in dung removal and secondary seed dispersal

Dung as a microhabitat

Dung pats lying scattered as dung islands in the animal's foraging area can be regarded as separate ecological units or microhabitats (Elton, 1949; Mohr, 1943). Due to their ephemeral and patchy distribution, dung pats are rather uncertain food sources and are therefore often places of fierce competition between species exploiting this resource. Dung fauna can be extremely abundant in some cases. One of the most extreme examples found in literature is the observation of Anderson and Coe (1974) who counted approximately 16 000 dung beetles on a 1.5 kg heap of elephant dung in East Africa. These beetles ate, buried and rolled this minor habitat away in less than two hours. Competition is not the only factor shaping the dung insect community, but it certainly is one of the major drivers (Hanski and Cambefort, 1991b). In fact, dung pats can be seen as some kind of islands made of high-quality resources lying in the matrix of surrounding vegetation. These islands can be highly variable in size and composition, but they share several specific attributes: scattered spatial occurrence, relatively small size, and short existence or durational stability which is generally no longer than one insect generation (Hanski and Cambefort, 1991b). The unpredictable occurrence of dung pats in time and space generally results in high mobility and diversity of the associated fauna.

Complex insect communities can be found in dung pats which comprise several trophic levels, including coprophages, mycophages, saprophages, predators and parasites (Figure 1.4 and Table 1.1). The colonization of fresh dung pats consists of several stages (Mohr, 1943). In a first colonization wave, pioneer flies and dung beetles make excavations and tunnels in the dung. Later when the dung starts to dry out and becomes less suitable for dung beetles, dung pats become increasingly colonized by fungi (Harper and Webster, 1964), nematodes (Sudhaus, 1981), earthworms (Gittings et al., 1994), flies (Mohr, 1943), beetles (Koskela and Hanski, 1977) and mites (Glida and Bertrand, 2002). In some specific cases, even vertebrate species such as frogs are found living inside or underneath dung pats (Campos-Arceiz, 2009). In the later phases of dung degradation predation becomes increasingly relevant (Hanski and Cambefort, 1991b; Skidmore,

1991). In some species complex feeding strategies exist. For example, the yellow dung fly *Scatophaga stercoraria*, one of the most abundant dung insects in the northern hemisphere, has coprophagous larvae but in the adult phase it has a complex diet of nectar and the larvae of dung flies and beetles (Hanski and Cambefort, 1991b). Furthermore, parasites may have a profound effect on the activity and species richness of dung fauna. For instance, parasitic nematodes (e.g., *Physocephalus sexalatus*) which usually infect livestock use dung beetles as intermediate hosts. Although these parasites are seldom lethal for the beetle, infections may modify the beetles' behaviour and result in slower and less dung consumption and in smaller brood balls being buried at shallower depth (Boze, 2007). Many other arthropod groups are found in cattle dung, but are rarely considered as true members of the dung community (Floate, 2011). Such species are most often found in the later stages of dung degradation and are rather casual visitors from adjacent habitats such as typical detritus feeders which are casually found in rich organic soils or decaying plant material, or predators and parasites searching for prey or hosts (Floate, 2011). Typical examples of such species are centipedes (Chilopoda), woodlice (Isopoda), millipedes (Diplopoda), harvestmen (Opiliones), spiders (Araneae), earwigs (Dermaptera), springtails (Collembola), ants (Formicidae), click beetles (Elateridae), ground beetles (Carabidae), and true bugs (Hemiptera).

Although dung insect communities of tropical and subtropical regions generally contain more species of large dung beetles than temperate communities, it is not uncommon to encounter fewer species of the other representatives of the dung insect community in (sub)tropical regions (Hanski and Cambefort, 1991b). One possible hypothesis for this pattern is the high density of large dung beetle species in (sub)tropical regions. The rapid dung removal by large dung beetles could result in reduced resource availability for dung breeding flies. Another explanation for the low diversity of dung flies in the (sub)tropics is the high desiccation rate of dung pats. Furthermore, the low diversity and abundance of dung flies may result in a lower diversity of their predators (e.g., staphylinid, histerid and hydrophilid beetles) (Hanski and Cambefort, 1991b).

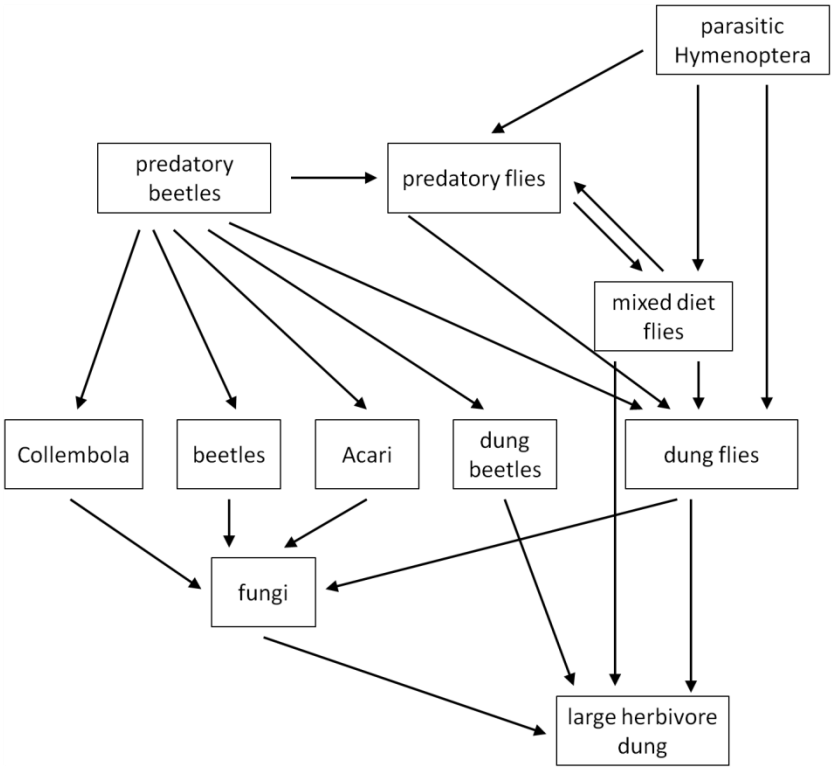


Figure 1.4 - Simplified food web of the insect community inhabiting cattle dung in Europe (modified from Hanski and Cambefort (1991b) and Floate (2011)).

Defining dung beetles

In literature, dung beetles are often defined as the species of the superfamily Scarabaeoidea that feed on dung in both the larval and adult phase. Some species of other beetle families such as the Hydrophilidae and Staphylinidae are commonly found feeding on dung and could, therefore, also be considered as dung beetles (Davis, 1990; Hanski and Cambefort, 1991b) (Table 1.1). However, most of these Hydrophilidae and Staphylinidae species are not coprophagous during their entire lifecycle and even have larvae that predate on e.g., Scarabaeoidea larvae (Finn et al., 1999). Furthermore, as these species do not contribute to the lateral or vertical transport of dung, and therefore have a limited impact on dung degradation, dung beetles were in this study strictly defined as the

coprophagous species in the Scarabaeoidea superfamily. This superfamily comprises around 35 000 species with approximately 200 new species described every year (Ratcliffe, 2002), and, recently, multigene-based phylogenetic approaches have redefined the classification of certain dung beetle groups (Tarasov and Dimitrov, 2016). Although dung beetles have a worldwide distribution with the exception of Antarctica (Davis et al., 2002), the greatest densities and biodiversity of dung beetles is found in the tropical and subtropical biomes (Cambefort, 1991). As a result, the majority of research papers focus on these regions or, due to their value for ecosystem functioning, on agricultural landscapes and microcosms.

Table 1.1 - General list of insect families which taxa feed and/or breed in dung microhabitats (original source Hanski and Cambefort (1991b), updated to current taxonomy). Taxa that are most often numerically or functionally dominant are in bold typeface.

food-web position	Coleoptera	Diptera	Hymenoptera
coprophages	**Geotrupidae**	Anthomyiidae	
	Hydrophilidae	Ceratopogonidae	
	Scarabaeidae	Chironomidae	
	Staphylinidae	**Muscidae**	
		Psychodidae	
		Lauxaniidae	
		Scatopsidae	
		Sciaridae	
		Sepsidae	
		Sphaeroceridae	
		Stratiomyidae	
mycophages	Cryptophagidae		
	Ptiliidae		
saprophages	**Staphylinidae**		
predators	Carabidae	Muscidae	
	Histeridae		
	Hydrophilidae		
	Staphylinidae		
parasitoids	Staphylinidae	Bombyliidae	**Braconidae**
			Eucoilidae
			Ichneumonidae
			Pteromalidae

Functional groups of dung beetles

Dung beetles make use of the liquid component of mammalian dung, typically containing a high concentration of micro-organisms, for feeding in both the adult and larval phase (Holter, 2000; Holter and Scholtz, 2005; Holter and Scholtz, 2007; Holter et al., 2002). As different dung manipulation and nesting strategies have evolved, dung beetles are traditionally classified into three functional groups (Doube, 1990; Halffter and Edmonds, 1982). Dwellers (endocoprids) feed and nest within a dung pat without moving it, while tunnelers (paracoprids) relocate dung in a vertical direction by digging tunnels of varying depth directly beneath the dung pat, and rollers (telocoprids) move dung in a combined horizontal and vertical direction by rolling balls of dung away from the dung pat and burying them shallowly (Figure 1.5). Tunneling species include all species in the Geotrupidae, some Scarabaeinae species (*Caccobius*, *Copris*, *Euoniticellus*, *Euonthophagus*, *Onitis*, *Onthophagus*) and some Aphodiinae species (*Colobopterus erraticus* in particular, see Rojewski (1983) and Vitner (1998)) while rollers are the remaining Scarabaeinae species (*Canthon*, *Gymnopleurus*, *Scarabaeus*, *Sisyphus*) and dwellers comprise almost all Aphodiinae species. Furthermore, the nesting structure differs between species, with different depths, nest complexity and number of broodballs per brood chamber in paracoprid and telocoprid species and a different zonation of nests within the dung pat for endocoprid species (Figure 1.5). In many studies of the dung removing activities of dung beetles a further classification is made according to size (e.g., Slade et al. (2007)) as the amount of dung transport and the depth of tunnels is strongly correlated with beetle size (Gregory et al., 2015; Horgan, 2001).

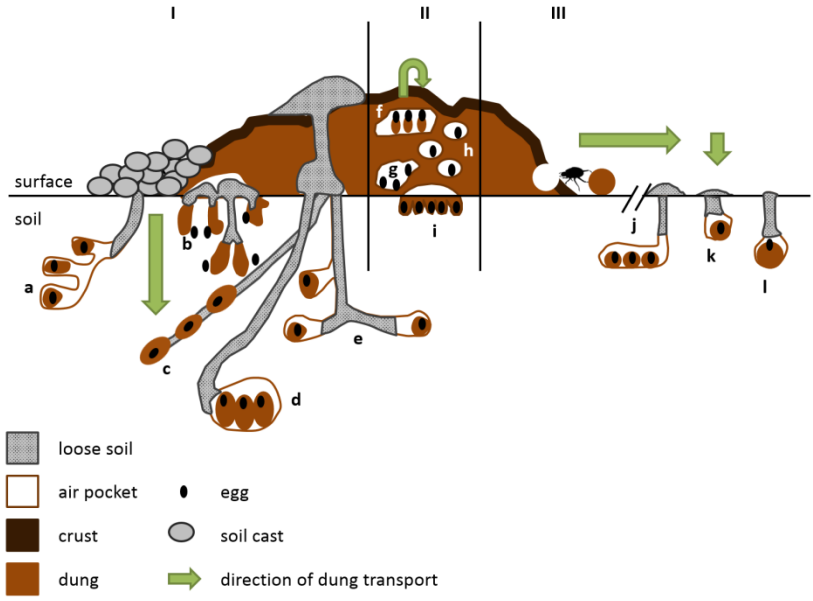


Figure 1.5 - Cross section through a dung pat and underlying soil depicting three nesting types: tunnelers (I), dwellers (II) and rollers (III). The main direction of dung transport is shown for each nesting type (modified from Bertone et al. (2006)). Different subtypes can be distinguished with a: complex multi-chamber tunnelling system (e.g., *Typhaeus typhoeus*), b: shallow nest (e.g., *Colobopterus erraticus*), c: tunnel with different brood masses (e.g., some *Onthophagus* species), d: multiple brood balls in one brood chamber (e.g., some *Copris* species), e: deep and complex burrow often made by *Geotrupes* species, f: nest in top-layers of dung containing multiple eggs (e.g., some *Aphodius* species), h: excavations containing 1 single egg (e.g., some *Aphodius* species), g: nest in middle layer of dung with multiple eggs (e.g., some *Aphodius* species), i: nest at the interface between dung and soil (some *Aphodius* species), j: roller nest containing multiple balls (e.g., some *Canthon* species), k: shallow roller nest (e.g., some *Sisyphus* species), and l: profound roller nest (e.g., some *Scarabaeus* species).

Although the life cycle of dung beetles is more or less identical (Figure 1.6), the duration of each stage differs greatly between functional groups and species. Due to the rather brief existence of dung pats, dweller species generally need less time to complete the larval stage compared to rollers and tunnelers. For instance, while the dweller species *Aphodius rufipes* is able to complete the larval stage in 40 to 55 days, the tunneler species *Typhaeus typhoeus* needs up to two years to complete this phase (Brussaard, 1985).

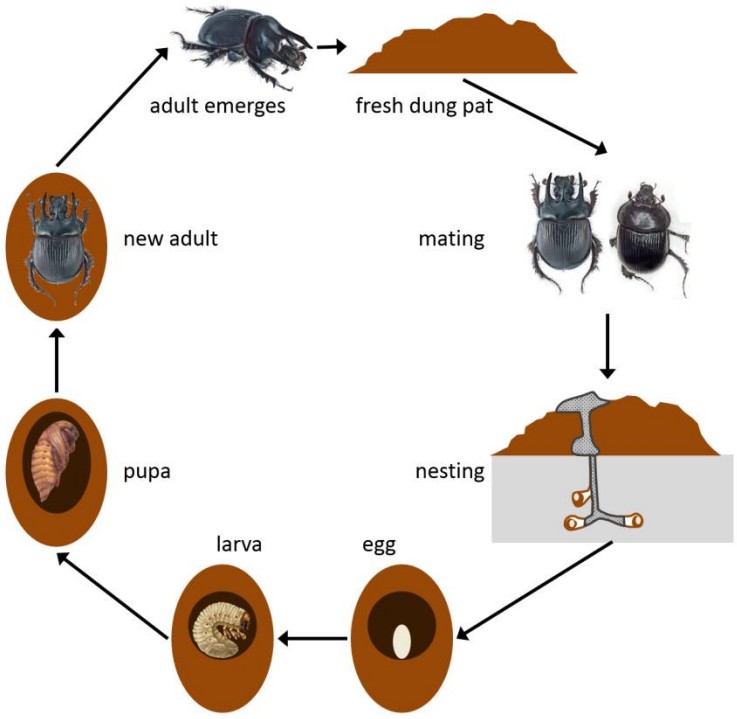


Figure 1.6 - Consecutive steps in the life cycle of the tunnelling dung beetle *Typhaeus typhoeus* (modified from Brussaard (1985)).

Spatial patterns of dung beetle assemblages

On a large spatial scale, the functional composition of dung beetle assemblages is highly variable between biogeographic and climatic zones (e.g., the differentiation found in tropical, subtropical, semi-desert rangeland, warm temperate, cold temperate and boreal ecosystems, and in wet versus dry conditions), habitat, altitude and dung availability (Hanski and Cambefort, 1991b; Menéndez and Gutiérrez, 1996; Scholtz et al., 2009). Even at a smaller geographic level, such differences are found as there is, for instance, a clear shift in dweller dominated assemblages in the northern temperate climate zone to increasing numbers of tunnelers and rollers in the southern temperate zone of the northern hemisphere (Hanski and Cambefort, 1991b). Furthermore, species richness and ecological strategy of dung beetles differ among climate zones and latitudes. The few species present in northern areas are mostly generalists, whereas dung beetle assemblages to the south are richer, more variable and contain more specialist species (Hortal et al., 2011). Furthermore, the competitive pressure differs between latitudes. Compared to tropical and subtropical dung beetle assemblages, the level of competition between the different dweller species in the northern regions is less intense and could be related to the fact that the cumulative larval consumption is only a fraction of the dung pat (Holter, 1982). Dung beetle assemblages in northern regions are hence rather determined by abiotic factors and predators (Hanski and Cambefort, 1991a) than by interspecific competition. In contrast, dung beetle assemblages of south temperate regions in the northern hemisphere are largely affected by competition for the scarce resource of dung which has led to the evolution of nesting strategies specialized in the rapid removal of dung by rolling and burying dung balls or by making underground tunnel systems (Hanski and Cambefort, 1991b).

On a local scale, environmental factors, habitat types, the availability of dung and human impacts such as farming and hunting largely defines dung beetle diversity and abundance. Whereas temperate European dung beetles are most attracted to dung from large domestic grazers such as cattle, horses and sheep, this is less so in temperate America where dung beetles are rather specialized in the niches of small mammal dung (Hanski and Cambefort, 1991b). Furthermore, modern agricultural management has a large impact on dung beetle diversity and abundance. In an assessment of

the impact of farm management, Hutton and Giller (2003) found lower abundance, biomass, diversity and species richness in intensive and rough grazing farms compared to organic farms[1]. Although the abandonment of pastoral practices are known bottlenecks for dung beetle diversity (Barragán et al., 2011; Carpaneto et al., 2007; Lobo, 2001; Roslin and Koivunen, 2001), the intensification of agriculture and the use of veterinary drugs has led to a further decline in dung beetle diversity and abundance (Barbero et al., 1999; Negro et al., 2011).

Ecological functions of dung beetles

Most of the ecosystem functions provided by dung beetles are linked to the removal and underground burial of dung (Losey and Vaughan, 2006; Nichols et al., 2008), which results in bioturbation increase and enhanced nutrient cycling. While digging their nests, dung beetles perturb the soil, aerate it, change the hydrological properties (Brown et al., 2010) and altogether alter the structure of the soil top layers (Bang et al., 2005a). In Western Europe for instance, the presence of the tunnelling species *Typhaeus typhoeus* on its own is estimated to move 450 kg of soil per hectare annually (Brussaard and Visser, 1987). Dung beetles play an important role in nutrient cycling as they spread and bury vertebrate faeces containing considerable amounts of nutrients (Hanski, 1987), which increases the availability of these nutrients in the ecosystem (Yamada et al., 2007), plant growth (Borghesio et al., 1999) and net primary production (Miranda et al., 2000). Furthermore, the burial of dung leads to a reduction of the amount of volatilization of the greenhouse gas methane (Penttilä et al., 2013) and NH_3 (Steinfeld et al., 2006) and indirectly increases the nitrogen content of soils (Yokoyama et al., 1991). Another indirect effect of dung beetle activity is their role as pest control agents. Dung plays an important role in the lifecycle of many parasites infecting vertebrates, such as flatworms and nematodes, but also attracts external pest species such as flies. Burying dung indirectly results in the suppression of the development of pest species such as dung flies

[1] Hutton and Giller (2003) distinguish three types of grazing farms: intensive (intense anthelmintic treatment of cattle and high input of synthetic fertilisers and pesticides), rough grazing (some anthelmintic treatment of cattle and low imput of synthetic fertilisers and pesticides) and organic farms (no anthelmintic treatment of cattle and no input of synthetic fertilisers and pesticides).

(Bryan, 1973) and the reduction of the transmission of gastro-enteric parasites (Fincher, 1973; Fincher, 1975). The role of dung beetles as pest control agents even led to the introduction of Mediterranean and African dung beetles in Australia and North America where the native dung beetle fauna was unable to remove the vast amounts of dung produced by cattle farming (Bornemissza, 1976; Fincher, 1981; Fincher and Woodruff, 1975; Walters, 2008). Overall, a great economic value has been attributed to dung beetles. Although it is difficult to determine an exact value, the annual economic value of the services of dung beetles in forage production, dung removal, and parasite suppression has been estimated to at least 380 million dollars in the United States. Losey and Vaughan (2006) acknowledged that this value most probably is an underestimate while Beynon et al. (2015) have put a value of 367 million pounds on dung beetle ecosystem services in the United Kingdom alone.

Next to these ecosystem functions with direct economic benefits, dung beetles play an important role in plant ecology through the process of secondary seed dispersal and by changing the seed's micro-environment (Andresen, 2002b; Feer, 1999). Dung beetles can act as secondary dispersers of the seeds already present in dung and reshape plant communities (Andresen, 2003; Nichols et al., 2008). Secondary seed dispersal by dung beetles may result in a higher seedling establishment by reducing the risks of predation and mortality, directing dispersal to more favourable locations for germination and decreasing scramble competition for space and nutrients by seedlings (Andresen, 1999; Andresen and Feer, 2005; Andresen and Levey, 2004; Nichols et al., 2008). Once seeds are buried two post-dispersal seed fates are possible: germination or death. One of the main determinants whether seeds are germinable following secondary dispersal is the depth at which they are buried, which differs between dung beetle species (D'hondt et al., 2008) and the specific germination requirements of the plant species (Bliss and Smith, 1985; Limón and Peco, 2016).

Species composition and dung beetle functional group richness strongly affect key ecological functions such as dung removal and decomposition (O'Hea et al., 2010; Slade et al., 2007), secondary seed dispersal of seeds already present in dung (Slade et al., 2007) and subsequent seed germination (D'hondt et al., 2008). Due to their broad geographic distribution, the ease of assigning species to functional groups and manipulating the functional diversity, and the knowledge that most of their

ecosystem functions can be estimated by measuring the removal rate of dung, dung beetles are often used as focal organisms in many biodiversity, conservation and ecosystem functioning studies (Spector, 2006).

Given the latitudinal differentiation of dung beetle functional groups and the expected effects of climate change on temperature, one can also expect a change in dung beetle community functionality over time, with an expected increase of tunneler and roller presence to the north (on the northern hemisphere). This may lead to a change in ecosystem functioning of local dung beetle communities.

1.2 Aims and objectives

Endozoochory has been a relatively popular research item in recent decades (see e.g., the PhD theses of Bonn (2004), Cosyns (2004), D'hondt (2011) and Mouissie (2004) for an extensive overview of endozoochory research in Belgium, Germany and the Netherlands). Most of these previous studies studied the first steps of the seed dispersal cycle: seed uptake and the excretion of viable seeds (Figure 1.2, processes marked in red). They, therefore, provided extensive proof for the existence and relevance of endozoochorous dispersal of dry-fruited species by large herbivores in temperate ecosystems. Less is known about the next steps in the seed dispersal cycle, *i.e.* the post-dispersal fate of seeds (Figure 1.2, processes marked in green). Although the main prerequisite for endozoochory is the excretion of viable seeds, endozoochory can only be considered as a successful dispersal strategy if germination, seedling establishment, growth and flowering can take place at the deposition site. Therefore, the aim of this thesis is to fill some gaps in the knowledge of the post-dispersal fate of seeds and the effects of dung on the germination, establishment, growth and flowering of temperate grassland species present in dung. Furthermore, I expected to find at least some effects of the high densities in which seeds are deposited through inter- and intraspecific competition between seedlings growing in dung pats. In order to exclude external factors, many experiments were performed in controlled environments in the laboratory and greenhouse. Nevertheless, field observations may provide valuable information as well. Some species have shown an increasing distribution following the introduction of large grazers while the underlying mechanisms

remain unknown. A remarkable example of such a grazer increaser species is *Helianthemum nummularium* (L.) Mill. which was used as a model species in field and laboratory experiments. Furthermore, the potential for secondary seed dispersal by dung beetles is largely unknown for temperate regions, despite a considerable number of publications covering tropical and subtropical biomes. As this insect group is vulnerable to changes in land use and climate, large-scaled field experiments were conducted in order to provide more insight on the effects of differing dung beetle assemblages in dung removal and secondary seed dispersal in the Western Palaearctic.

More specifically, the objectives of this thesis are to study:

- the effects of the (simulated) environment of and passage through the gastrointestinal tract on germination;
- germination, establishment, growth and flowering in a dung environment;
- the existence and the effects of inter- and intraspecific competition on germination, establishment, growth and flowering, and the possible interaction between the outcome of competitive processes and the presence of dung;
- the zoochorous dispersal potential of the grazer increaser species *H. nummularium*;
- the effects of dung beetle assemblages and functional group composition on the ecosystem functions of dung removal and secondary seed dispersal, at different spatial scales.

1.3 General outline of the thesis

The thesis is divided into two main sections: the fate of seeds during and following endozoochorous dispersal by large herbivores (1) and the role of dung beetles in secondary seed dispersal and dung removal (2) (Figure 1.7).

In the first section, several aspects of seed fate during and following zoochorous dispersal are studied. The effects of herbivore digestion and the presence of dung on processes of germination, seedling establishment, growth and flowering were experimentally assessed. A comparison was made between the effects of endozoochorous dispersal and unassisted dispersal (chapters 2-5), and endozoochorous, epizoochorous and unassisted dispersal (chapter 6). More specifically, the following topics were studied:

In **chapter 2** the germination of grassland species was experimentally assessed using treatments simulating the gastrointestinal environment of cattle and horses. Using mechanical, chemical and thermal treatments, the effects of mastication, the chemical composition of stomach fluids and body temperature were estimated using 15 grassland species commonly associated with endozoochorous dispersal. In control treatments the comparison with unassisted dispersal was made.

In **chapter 3** the germination timing and probability of 15 annual and perennial grassland species was experimentally tested in horse and cattle dung. In a dung-free control treatment, the comparison was made with unassisted dispersal. Furthermore, the experiment was replicated in two different climatic conditions, in an artificial greenhouse environment and in outdoor conditions, in order to compare germination between the standardized artificial environment and field conditions.

In **chapter 4** seedling establishment, growth and flowering in cattle and horse dung was assessed using 12 temperate grassland species with a known potential for endozoochory. Seedling biomass and growth rate, height, ramification, flowering and biomass of grown plants were measured in attempt to quantify the benefits of

endozoochorously dispersed seeds compared to seeds dispersed by other means growing in a dung-free environment.

In **chapter 5** the effects of inter- and intraspecific competition on the establishment, growth and flowering of three common perennial grassland species, known to germinate from dung, were experimentally tested in two-species combinations in different proportions and seed densities. As in the previous chapters, the comparison was made between endozoochorous dispersal by cattle and horses by using dung as a sowing substrate, while unassisted dispersal was studied in a dung-free control treatment.

In **chapter 6** the zoochorous dispersal potential of *Helianthemum nummularium* was studied. As this species has shown a considerable increase in its distribution since the introduction of large domestic ungulates in dune grasslands on the Belgian coast, both the potential for endozoochorous and epizoochorous dispersal were estimated in several aspects of fruit removal, seed transfer, germination, seedling establishment, growth and fruit production.

In the second part the impact of dung beetles on the ecosystem functions of dung removal and secondary seed dispersal was measured at different spatial scales (regional and continental).

In **chapter 7** the relation between dung beetle functional group richness and the ecosystem functions of dung removal and secondary seed dispersal was experimentally quantified on a regional scale. Using exclusion treatments dung removal by different functional groups was measured in two different habitats in Belgium (coastal grassland and inland heathland) and during two consecutive seasons in the same year.

In **chapter 8** a comparable experiment was run as in chapter 7, but on a much broader, continental scale. The effects of the local dung beetle assemblages and functional group richness were quantified by measuring dung removal and secondary seed dispersal in 17 study sites located along a biogeographic and climatic gradient.

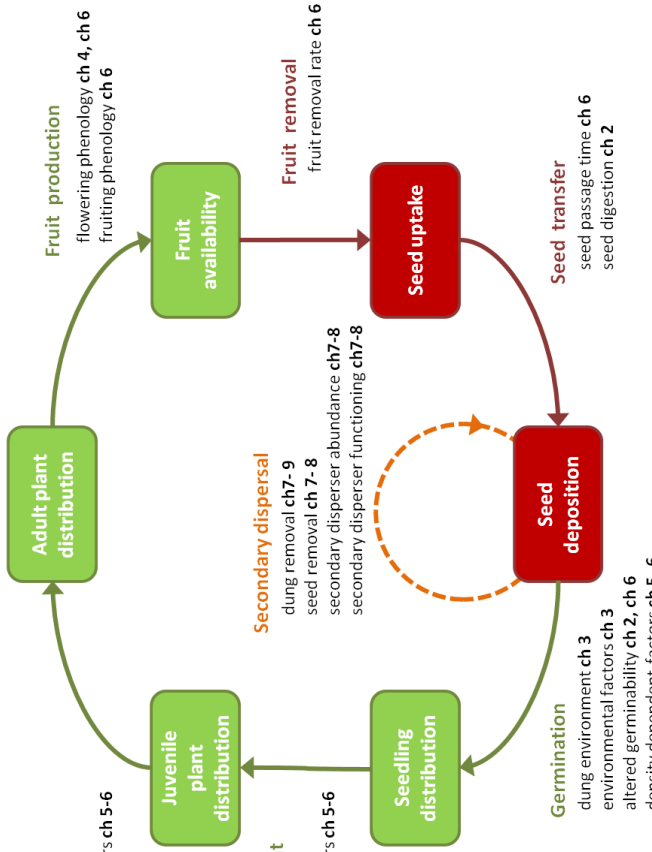


Figure 1.7 - Schematic outline of the thesis using the seed dispersal cycle from Figure 1.2. Different aspects determining endozoochorous seed dispersal success are listed with the chapters discussing these factors. In chapters 1 to 5 particular aspects of the germination, seedling establishment and/or growth and maturation phase are discussed while in chapter 6 determinants of the whole cycle are used in a comparison with epizoochorous dispersal success. In chapters 7 and 8 the effects of dung beetles as secondary seed dispersers are studied.

Plant species used in the experiments

23 plant species belonging to 9 plant families were used in the various experiments in this thesis (Table 1.2). Most of the species have a known link with endozoochory (see Table 1.3, and Cosyns' (2004) extended list of species germinating from dung sampled in temperate grasslands) and are relatively common in temperate European grasslands. Special attention has been given to *Helianthemum nummularium* (e.g., chapter 6) as the abundance of this normally rare species has remarkably increased since the introduction of large herbivores in calcareous dune grasslands in Belgium (Lejeune and Verbeke, 2009; Provoost et al., 2011a). As the set-up of some of the experiments (e.g., chapter 2) included the comparison of life strategies (annual versus perennial species) within the same plant family, some exceptions were made. For instance, *Tuberaria guttata* was included in the experiments due to the absence of annual Cistaceae species in the Belgian flora, while its main distribution area lies in the Mediterranean region (Herrera, 2004) apart from some scattered and historic localities in the United Kingdom, Germany and the Netherlands (Proctor, 1960). Furthermore, *T. guttata* has a close link to endozoochorous dispersal as its seeds have been found germinating from dung in Mediterranean dehesas (Malo and Suárez, 1995b; Malo and Suárez, 1996). *Cistus albidus* is exclusively found in warm and sunny climates such as the Mediterranean region (Thanos et al., 1992) and it is not known whether its seeds are dispersed by endozoochory (although the congeneric species *Cistus ladanifer* has been found in dung from Mediterranean dehesas (Malo and Suárez, 1995b)). This species has been selected for the experiments in chapter 2 as it is a Cistaceae species and its seeds were available for purchase in a specialised web shop (which is not the case for most wild Cistaceae species). In Table 1.2 additional details regarding life form, ecological strategy, nitrophily and grazing tolerance are listed. Although these traits certainly affect the probability of being consumed by herbivores and the establishment success in dung, plant species were not specifically selected based upon these traits. The nature of the diaspores used in the germination and secondary dispersal experiments are listed in Table 1.3. For the experiments studying secondary dispersal by dung beetles, three plants species were selected according to diaspore dimension: elongated and large (*Alopecurus myosuroides*), spherical and medium-sized (*Galium aparine*), and elongated and small (*Poa pratensis*).

Table 1.2 - List of the plant species used in the experiments with indication of life strategy, life form (with Cp= chamaephyte, Gp= geophyte, hCp= hemicryptophyte, hPp= hemiphanerophyte, and Tp= therophyte), ecological strategy as defined by Grime (1977) (with C= competitors, R= ruderals, S= stress-tolerators and the intermediate types CR, CS, SR and CSR), nitrophily based on the nitrogen indicator values defined by Ellenberg (1974) using an ordinal scale ranging from 1 (soils extremely low in N) to 9 (soils extremely rich in N) (and with x= indifferent), grazing tolerance as defined in the BiolFlor database (Kühn et al., 2004) which uses ordinal scale ranging from 1 (grazing intolerant) to 9 (very tolerant to grazing) (www.ufz.de/biolflor, accessed January 2017), and the chapters in which the respective species were used. *na*= data not available.

plant family and species name	life strategy	life form	ecological strategy	nitrophily	grazing tolerance	used in chapter						
						2	3	4	5	6	7	8
Caryophyllaceae												
Stellaria media (L.) Vill.	annual	hCp, Tp	CR	7	4		x					
Cistaceae												
Cistus albidus L.	perennial	hPp	*na*	*na*	*na*	x						
Helianthemum nummularium (L.) Mill.	perennial	hPp	CS	2	6	x	x	x		x		
Tuberaria guttata (L.) Fourr.	annual	Tp	SR	1	*na*	x	x	x				
Cyperaceae												
Carex acuta L.	perennial	Gp	CS	4	3	x						
Carex flacca Schreb.	perennial	Gp	CSR	x	6	x						
Fabaceae												
Medicago arabica (L.) Huds.	annual	Tp	R	5	*na*	x						
Medicago lupulina L.	perennial	hCp, Tp	CSR	x	4	x						
Trifolium arvense L.	annual	hCp, Tp	SR	1	3	x						
Trifolium campestre Schreb.	annual	hCp, Tp	R	3	4	x						
Trifolium pratense L.	perennial	hCp	C	x	4	x	x	x	x	x		
Trifolium repens L.	perennial	hCp	CSR	6	8	x	x	x	x	x		

Table 1.2 continued.

plant family and species name	life strategy	life form	ecological strategy	nitrophily	grazing tolerance	used in chapter 2	3	4	5	6	7	8
Gentianaceae												
Centaurium erythraea Baumg.	perennial	Cp	CSR	6	4		x					
Juncaceae												
Juncus bufonius L.	annual	Tp	R	4	5		x	x				
Juncus effusus L.	perennial	hCp	C	4	7		x	x				
Poaceae												
Agrostis capillaris L.	perennial	hCp	CSR	4	5		x	x				
Agrostis stolonifera L.	perennial	hCp	CSR	5	9		x	x	x	x		
Alopecurus myosuroides Huds.	annual	hCp, Tp	R	6	*na*		x	x			x	x
Poa annua L.	annual	hCp, Tp	R	8	9	x	x	x				
Poa pratensis L.	perennial	hCp	C	6	8	x	x	x			x	x
Rubiaceae												
Galium aparine L.	annual	hCp, Tp	CR	8	2						x	x
Urticaceae												
Urtica dioica L.	perennial	Cp, hCp	C	8	8	x	x	x				
Urtica urens L.	annual	Tp	R	8	8	x	x					

Table 1.3 - Dispersal traits of the selected species. Fruit, diaspore and germinule types (with Ac= achene, Cap= capsule, Car= caryopsis, F= fruit, Fa= fruit with appendages, Leg= legume, MC= mericarp, SC=schizocarp, S= seed), and the diaspore types used in the experiments with minimum and maximum diaspore mass ('m'), length ('l'), width ('w') and height ('h') as defined in the BiolFlor database (www.ufz.de/biolflor, accessed January 2017), number of dispersal vectors, known dispersal methods (with A= autochory, An= anemochory, Ch= chamaechory (specific type of anemochory with diaspores rolled along the ground surface by wind), Endo= endozoochory, Epi= epizoochory, H= hemerochory (generic category for all seed dispersal related to human activities), Hy= hydrochory) as defined in the LEDA-database (Kleyer et al. (2008), www.leda-traitbase.org, accessed January 2017). *na*= data not available.

				diaspore used in the experiments					known dispersal methods	
plant family and species name	**fruit**	**diaspore**	**germinule**	**type**	**m (mg)**	**l (mm)**	**w (mm)**	**h (mm)**	**# vectors**	**methods**
Caryophyllaceae										
Stellaria media	Cap	S	S	S	0.3-0.7	0.8-1.5	0.7-1.4	0.4-0.8	5	A, Endo, Epi, H, Hy
Cistaceae										
Cistus albidus	Cap	*na*	*na*	S	*na*	*na*	*na*	*na*	*na*	
Helianthemum nummularium	Cap	S	S	S	0.7-1.3	1.2-2.0	0.8-1.6	0.6-1.2	5	A, Endo, Epi, H, Hy
Tuberaria guttata	Cap	S	S	S	0.1	0.6	*na*	*na*	2	Endo, Epi
Cyperaceae										
Carex acuta	Ac	Fa	F, Fa	Ac	0.5-0.6	2.0-3.2	1.4-2.0	0.5-0.9	4	A, An, Epi, Hy
Carex flacca	Ac	Fa	F, Fa	Ac	1.1	2.0-3.1	1.0-1.6	1.0-1.4	6	A, An, Endo***, Epi, H, Hy
Fabaceae										
Medicago arabica	Leg	Fa	Fa, S	S*	1.6-1.7	2.4-3.5	1.2-1.7	0.6-0.8	2	Endo, Epi
Medicago lupulina	Leg	Fa	Fa, S	S*	1.4-1.8	1.2-2.3	0.8-1.8	0.5-1.2	5	A, Endo, Epi, H, Hy
Trifolium arvense	Leg	Fa	Fa, S	S*	0.3-0.6	0.8-1.5	0.6-1.0	0.4-0.8	6	An, Ch, Endo, Epi, H, Hy
Trifolium campestre	Leg	Fa	Fa, S	S*	0.2-0.5	0.9-1.6	0.5-1.0	0.3-0.8	5	An, Endo, Epi, H, Hy
Trifolium pratense	Leg	Fa	Fa, S	S*	1.0-2.2	1.3-2.4	1.0-1.8	0.7-1.3	5	An, Endo, Epi, H, Hy
Trifolium repens	Leg	Fa	Fa, S	S*	0.4-0.8	0.8-1.5	0.6-1.3	0.4-1.0	4	Endo, Epi, H, Hy

Table 1.3 continued.

				diaspore used in the experiments					known dispersal methods	
plant family and species name	**fruit**	**diaspore**	**germinule**	**type**	**m (mg)**	**l (mm)**	**w (mm)**	**h (mm)**	**# vectors**	**methods**
Gentianaceae										
Centaurium erythraea	Cap	S	S	S	0.1-0.2	*na*	*na*	*na*	6	A, An, Ch, Endo**, Epi, H
Juncaceae										
Juncus bufonius	Cap	S	S	S	<0.1	0.2-0.6	0.2-0.4	0.2-0.3	5	A, Endo, Epi, H, Hy
Juncus effusus	Cap	S	S	S	<0.1	0.2-0.5	0.1-0.4	0.1-0.3	5	A, Endo, Epi, H, Hy
Poaceae										
Agrostis capillaris	Car	Fa	Fa, F	Fa*	<0.1-0.1	1.4-2.5	0.3-0.6	0.3-0.5	5	An, Endo, Epi, H, Hy
Agrostis stolonifera	Car	Fa	Fa, F	Fa*	<0.1-0.1	1.5-2.5	0.4-0.5	0.4-0.5	5	An, Endo, Epi, H, Hy
Alopecurus myosuroides	Car	Fa	Fa, F	Fa*	2.0-2.8	4.0-7.5	1.4-2.3	0.5-1.1	5	An, Endo, Epi, H, Hy
Poa annua	Car	Fa	Fa, F	Fa*	0.2-0.5	2.0-4.0	0.7-1.3	0.5-0.9	5	A, Endo, Epi, H, Hy
Poa pratensis	Car	Fa	Fa, F	Fa*	0.2-0.4	2.2-4.0	0.6-1.0	0.3-0.8	4	Endo, Epi, H, Hy
Rubiaceae										
Galium aparine	SC	MC	MC	MC	5.7-9.9	1.9-7.0	1.9-4.0	1.5-3.5	4	Endo, Epi, H, Hy
Urticaceae										
Urtica dioica	Ac	Fa	Fa, F	Ac	0.1-0.2	0.8-1.5	0.5-1.0	0.2-0.5	5	An, Endo, Epi, H, Hy
Urtica urens	Ac	Fa	Fa, F	Ac	0.4-0.6	1.7-2.0	1.1-1.4	0.3-0.7	3	An, Endo, H

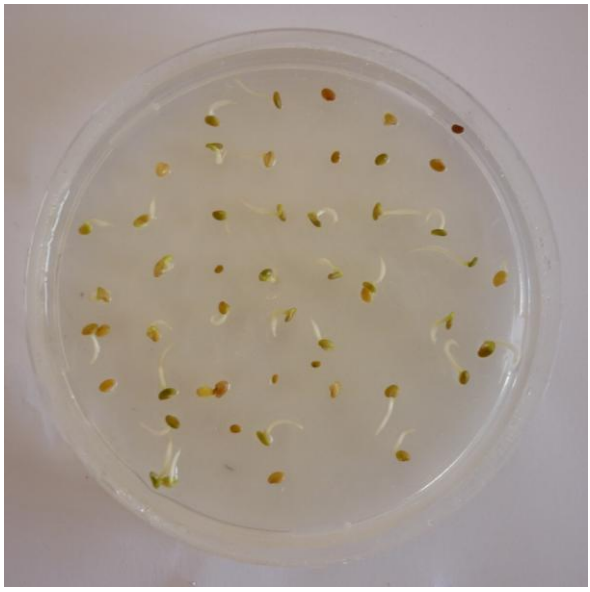
PART I: SEED FATE

The fate of seeds during and following endozoochorous dispersal

Seedlings in a decomposing cattle dung pat (autumn 2011, Vallei van de Oude Kale, Nevele, Belgium) ©Bastiaan Notebaert

2 How does gut passage impact endozoochorous seed dispersal success? Evidence from a gut environment simulation experiment

Tanja Milotić and Maurice Hoffmann



Trifolium repens seeds germinating on agar.

Modified from Milotić, T. and Hoffmann, M. (2016) How does gut passage impact endozoochorous seed dispersal success? Evidence from a gut environment simulation experiment. Basic and Applied Ecology, **17**(2), 165-176, DOI: 10.1016/j.baae.2015.09.007

Abstract

Endozoochory of temperate grassland species is a widespread phenomenon and may accelerate and/or increase germination in some plant species. However, the mechanisms causing this altered germination success are only partly understood. In this study, germination of common grassland species was evaluated after simulated herbivore digestion in a standardized laboratory environment. Ruminants (cattle) and hindgut fermenters (horses) were used as model organisms in this simulation experiment. Three major digestive processes were studied through mechanical, thermal and chemical treatments of the seeds simulating mastication, body temperature and digestive fluids, respectively. Congeneric groups of annuals and perennials were tested with 15 species belonging to the plant families Cistaceae, Cyperaceae, Fabaceae, Poaceae and Urticaceae. No differences between the impact of the simulated herbivore gut environments could be found, but major differences in germination behaviour were found among plant species. For most of the tested plant species, treatments had a decelerating and inhibiting effect on germination compared to the untreated seeds. However, species of the Cistaceae and Fabaceae benefitted from mechanical treatments. Species of the Cyperaceae and Poaceae were hardly impaired by any of the treatments and even germinated better after chemical treatments. Thermal treatments, simulating the body temperature, inhibited germination in most cases. The germination success of *Urtica urens* was significantly higher after all treatments, which suggests seeds are specifically well adapted to gut passage, and hence to endozoochorous dispersal.

Keywords: endozoochory, germination, seed dispersal

Introduction

Seed dispersal is the link between the end of the reproductive cycle of the adult plant and the start of a new one by the establishment of offspring. It is one of the major drivers of vegetation composition as the process enables the colonization of new areas, maintains genetic diversity and has indirect implications on succession, regeneration and conservation (Wang and Smith, 2002). According to the Janzen-Connell hypothesis seed dispersal is indispensable to enable the escape of seeds and seedlings from high density-dependent mortality caused by pathogens, seed predators, and/or herbivores in the direct vicinity of the parent plant (Connell, 1971; Janzen, 1970). This implies that those seedlings that are growing farthest from conspecific adults have a survival advantage. Dispersal agents are either abiotic (wind and water) or biotic (animals and the plant itself) and are related to diaspore morphology (Levin et al., 2003). Animal mediated dispersal implies that seeds are attached externally (epizoochory) or ingested and dispersed in excreted dung (endozoochory). Many taxonomic groups have been reported to act as endozoochorous dispersers, including frugivorous birds (e.g., Wenny (2000)), ants (e.g., Clarke and Davison (2001)), beetles (de Vega et al. (2011)), rabbits (e.g., Malo and Suárez (1995b)), foxes (D'hondt et al. (2011)) up to the largest terrestrial herbivores (Campos-Arceiz and Blake (2011)). Endozoochory by large herbivores could be one of the main mechanisms of long distance dispersal as a vast number of germinable seeds of many grassland species can be found in dung (e.g., Cosyns et al. (2005a), Couvreur et al. (2005a), Malo et al. (2000), Pakeman et al. (2002)). Furthermore, the combination of large home ranges, high travel velocity, large gut capacity and long seed retention time enables seeds to travel several kilometres away from the parent plant (Cousens et al., 2010; Nathan et al., 2008; Pakeman, 2001).

The survival of seeds in the digestive system is one of the main determinants of successful endozoochorous dispersal. Seed feeding experiments have resulted in contrasting outcomes with an increased germination success in some cases (e.g., D'hondt and Hoffmann (2011), Grande et al. (2013), Mancilla-Leyton et al. (2011), Manzano et al. (2005), Ramos et al. (2006)) and reduced germination in others (e.g., Cosyns et al. (2005b), D'hondt et al. (2011), Grande et al. (2013), Manzano et al. (2005), Mouissie et al. (2005b)). Successful endozoochorous dispersal is known to

be related to both plant and herbivore traits (Albert et al., 2015b). Plant traits such as diaspore size and shape, permeability or thickness of the seed coat, seed longevity and seed production (Bruun and Poschlod, 2006; Cosyns and Hoffmann, 2005; Mouissie et al., 2005b; Pakeman et al., 2002), and herbivore traits such as diet, digestive system, seed retention time and body mass (Clauss et al., 2003; Cosyns and Hoffmann, 2005; Van Weyenberg et al., 2006) are important in determining whether seeds can be dispersed effectively through herbivore guts.

Mean retention time of seeds in the digestive system differs between ruminants and hindgut fermenters with generally longer mean retention times for horses than cattle (Cosyns et al., 2005b). During the endozoochorical process, seeds are subjected to a range of digestive actions. During ingestion and rumination, the seeds may be abraded or crushed by the grinding action of the teeth. Also, seeds are held inside a warm and wet environment equalling the body temperature of the herbivore. Additionally, seeds are subjected to a wide range of chemical processes. Proteolytic and cellulolytic enzyme secreting bacteria can become attached to the seed surface in the rumen and large intestine of cattle (Gardener et al., 1993b) and in the cecum and colon of horses (Householder et al., 1993), while in the abomasum or stomach and first part of the small intestine (duodenum), seeds are soaked in acid (pH 2-4) and exposed to proteolytic, amylolytic and lipolytic enzymes (Dijkstra et al., 2005; Gardener et al., 1993a; Gardener et al., 1993b).

In many studies, the endozoochorous dispersal potential of many plant species has been assessed experimentally by feeding a known number of seeds to herbivores or by germination trials of collected dung samples. However, many processes are influencing the germination success (e.g., density dependent germination, alternating environmental conditions, etc.) and can hardly be controlled in field conditions. In this study, the germination capacity and rate of common grassland species was tested after simulated herbivore digestion in a standardized laboratory environment. Ruminants (cattle) and hindgut fermenters (horses) were used as model organisms in this simulation experiment and three major digestive actions were studied through mechanical, thermal and chemical treatments simulating mastication, body temperature and digestive fluids. Congeneric pairs of grassland species from the Cistaceae, Cyperaceae, Fabaceae, Poaceae and Urticaceae which are known to be dispersed endozoochorically

were used in order to find family or genus-related responses to our treatments. We hypothesized that early germination in a highly competitive environment such as dung is beneficial for the survival and reproductive probabilities of the individual. We expected to find interspecific differences in germination rates and germinability, with faster and more germination in species which are frequently found germinating in dung samples (such as *Trifolium* and *Poa* species). Also, the mechanical treatments were expected to enhance germination in hard seeded species, such as the tested species in the Cistaceae and Fabaceae.

Materials and methods

Plant species

Seeds of 15 grassland species belonging to 5 different families were used in this germination experiment. In most congeneric couples within each family an annual and a perennial species were selected (see appendix, Table A 2.1 regarding selected species and seed origin). Exceptions were the Cyperaceae where no annuals were used, the Cistaceae with the selection of 2 perennial and 1 annual species and the 3 annual and 3 perennial Fabaceae species. Most of the species are common in temperate European grasslands and were previously found germinating in herbivore dung (Cosyns et al., 2006; Cosyns et al., 2005b; Cosyns and Hoffmann, 2005; Couvreur et al., 2005a). The Cistaceae species were included as adaptations to endozoochory were presumed. The abundance of the normally rare species *Helianthemum nummularium* remarkably increased after the introduction of large herbivores in calcareous dune grasslands in Belgium (Provoost et al., 2015) and the germination rate is known to increase after gut passage. The other species of the Cistaceae, *Cistus albidus* and *Tuberaria guttata*, have a Mediterranean distribution (Herrera, 2004; Thanos et al., 1992) and have been found to germinate from dung as well (Malo and Suárez, 1995b; Malo and Suárez, 1996).

Experimental treatments

The experiments were run during two periods of 100 days each. Five pre-germination treatment types were applied to the seeds, each representing steps in the digestive process in the gut system of horses or cattle (Appendix, Table A 2.1).

The first treatment group was the control group in which intact, untreated seeds were used as a reference to the other treatments (treatment "*control*").

In the second set of treatments, seeds were treated mechanically in order to simulate the mastication process. Three different methods were used. A first set of seeds was scarified using fine grit sandpaper with average particle diameter of 125 µm. Seeds were put between two pieces of sandpaper which were moved twice (treatment "*MS*"). A second set of seeds were scarified using a cattle skull with the original teeth (treatment "*MC*"). On average the mastication intensity for cattle is 3.61 chews/g dry matter (Janis et al., 2010), while Erlinger et al. (1990) calculated that cattle consume on average 0.57g of dry matter per bite. Combining both figures, seeds were chewed twice using a cattle skull. A third set of seeds was treated likewise using a horse skull (treatment "*MH*"). The average mastication intensity for horses is 4.61 chews/g dry matter (Janis et al., 2010) and according to Fleurance et al. (2009) the intake rate (*IR*) can be defined as follows:

$$IR = -0.1972 + 0.0055BM$$

where *BM* is the biomass of the grassland in g dry matter/m². Using an average biomass of temperate dry and medium nutrient-rich grasslands of 206 g DM/m² (Tahmasebi et al., 2008), we concluded that chewing seeds four times with a horse skull would be a good simulation of mastication in this experiment. As a measure for standardization for the pressure of the jaws, all mastication treatments were executed by one person.

A third group of treatments dealt with the chemical scarification of seeds. It simulates the chemical abrasion of the outer seed layers in the stomach. The pH of the mixture of food and digestion fluids in the abomasum of cattle and the stomach of horses is highly dependent on the forage and physiological status of the animal and ranges between pH 2 and 4 for cattle (Dijkstra et al., 2005) and between pH 1.5 and 4.6 for horses (Murray and Schusser,

1993), while the gastric retention time of ingesta ranges between 15 minutes and two hours for cattle (Dijkstra et al., 2005) and even up to six hours for horses (Van Weyenberg et al., 2006). For 15 minutes, a subset of seeds was put in a hydrochloric acid solution with pH 3.5 which falls within the range of previously reported concentrations of hydrochloric acid in the abomasum and stomach of respectively cattle and horses (treatment "*HCl*"). In the abomasum and stomach both hydrochloric acid and pepsinogen are secreted. When the pH drops below 4, the pepsinogen gets activated into pepsin which breaks down proteins into peptides and amino acids (Dijkstra et al., 2005). Therefore, a second and third set of seeds were put in a mixture of hydrochloric acid (pH 3.5) and 1% pepsin for 15 minutes (treatment "*HCl-P15*") or 60 minutes (treatment "*HCl-P60*"), following the Tilley and Terry method (Faithfull, 1984). All chemically treated seeds were rinsed with distilled water afterwards.

In the fourth group of treatments, the increased temperature during the passage through the intestinal system was simulated. Both cattle and horses have a constant body temperature of 38°C (Brown-Brandl et al., 2005; Green et al., 2005), while the retention time of seeds in the intestinal system differs among herbivore species. According to Cosyns (2004) germinating seeds were found after a mean retention time of 49 and 55 hours for cattle and horses, respectively, with a minimal and maximal retention time of 24 and 72 hours for cattle, and 24 and 96 hours for horses. Therefore, four thermal treatments were tested putting intact seeds in Eppendorf tubes filled with distilled water in a warm water bath at a constant temperature of 38°C for 24 h, 49 h, 55 h and 72 h (treatments *"T24", "T49", "T55"* and *"T72"* respectively).

In the fifth group of treatments, a combination was made of the mechanical, chemical and thermal treatments for cattle and horses separately. In the combination treatment for cattle (treatment "*combiC*") treatments MC, HCl-P15 and T49 were executed consecutively, while in treatment "*combiH*", the horse treatments MH, HCl-P15 and T55 were combined.

For all species at least one of the treatments within each treatment group was applied (Appendix, Table A 2.1). The same day the treatments were finished, 50 seeds per replicate were sown at equal distance in Petri dishes filled with 1% water agar. In order to prevent evapotranspiration, the Petri dishes were closed with Parafilm tape. Per treatment and per species five

replicates were used and all Petri dishes were arranged in a complete random design in a germination chamber with day: night fluctuating temperatures between 14 °C and 30 °C with a 12 h day: 12 h night period. Light was provided by white fluorescent tubes (Sylvania, 36 W, Germany) with a light intensity (photosynthetic active radiation, PAR) between 10 and 45 µmol/m²s depending on the location on the rack. During the first four weeks of the experiments, newly germinated seeds were counted five times per week, while the counting effort was lowered to once in every two weeks once the majority of seeds had germinated. After each seed counting, the Petri dishes were put back in a new randomized sequence in order to avoid interference with possible unequal abiotic conditions in the germination chamber. Germination was considered to occur whenever a white radical emerged through the testa. Germinated seeds were discarded after counting in order to prevent interference with the remaining seeds.

Cattle skull used in the mastication treatment.

Data analysis

After finishing the experiments, the count data were converted to germinability and mean time per germination (MTG). Germinability can be defined as the cumulative percentage of seeds that have germinated by the end of the experiment, while MTG was calculated as:

$$\mathrm{MTG} = \frac{\sum_{i=1}^{k} d_i * n_i}{N}$$

where d_i is time from the start of the experiment to the i^{th} observation, n_i is the number of seeds germinated within the period i, N is the total number

of germinated seeds during the experiment and *k* is the last time of germination (Ranal and Garcia De Santana, 2006).

As the data for both MTG and germinability are nested structures with each species belonging to one family, a nested ANOVA analysis was performed with MTG or germinability as measured variable and treatment, life strategy and family as nominal variables with a nested family: species term. In order to meet the assumptions of parametric tests, MTG and germinability data were respectively $\log_{10}$ and arcsine square-root transformed.

One-way ANOVAs were performed per species in order to gain insight in the treatment effects per species and Tukey post hoc tests were applied in order to distinguish between significantly differing treatments.

Recently, time-to-event or survival analysis was proposed as a more solid statistical method coping with the timing of germination (McNair et al., 2012; Onofri et al., 2010). The advantage of using these methods is that the timing of events (in this case germination) which occurs over time can be analysed including the effects of different factors (e.g., treatments). Our germination data are of the interval type with time intervals between two successive germination records varying between 1 and 14 days implying that the actual germination takes place between two successive germination observations. Seeds not germinated at the end of the experiment were considered as 'right-censored' observations as they were expected to germinate at an unknown moment after the end of the experiment.

The germination probability was estimated non-parametrically by the Kaplan-Meier method:

$$S(t) = \prod_{j=1}^{s} \left(1 - \frac{d_j}{n_j}\right)$$

where d_j is the number of seeds germinating in a given interval of time j, n_j is the number of seeds 'at risk' of germination in the same interval, which is the number of non-germinated seeds entering the interval minus one half of the number of seeds germinated during the same interval (Venables and Ripley, 2002). The germination probability was calculated for each treatment and species using the function *survfit* in the R environment.

Comparisons among germination curves were performed using AFT (accelerated failure time) regressions using the *survreg* function in R. Exponential, Weibull, Log-Normal and Log-Logistic distributions were tested per species. As suggested by Onofri et al. (2010) the problem of clustering of seeds within randomisation units (Petri dishes) was overcome by adopting a frailty approach with Petri dishes as the clustering (gamma distributed) random effect. The second order Akaike's information criterion (AICc) and a graphical comparison of the model outcome with the Kaplan-Meier estimator were used to select the best fitting model for each species.

Using the method of Onofri et al. (2010), time ratios were derived from the AFT models. In this method, the germination time after each treatment is compared to the control treatment which has a time ratio of 1. Treatments with a time ratio higher than 1 resulted in increased germination time, while treatments with a lower time ratio than the control treatment have lower germination times.

All analyses were performed with R version 3.1.2 (R Core Team, 2014) using the packages 'survival' version 2.38-1 and 'agricolae' version 1.2-1.

Results

Mean time to germination and germinability

Treatments had a significant effect on both the mean time to germination (MTG) and germinability (Table 2.1). Furthermore, MTG and germinability differed significantly between plant families and between species within families, while different life strategies had only an effect on germinability (Table 2.1).

Table 2.1 - Results of nested ANOVA analyses with consecutively mean time to germination (MTG) and germinability as measured variable and treatment, life strategy and family as nominal variables with a nested family: species term.

measured variable	factor	*df*	*F* value	*p*
MTG	treatment	12	6.614	<0.001
	life strategy	1	0.039	0.843
	family	4	62.994	<0.001
	family: species	9	30.7	<0.001
germinability	treatment	12	29.499	<0.001
	life strategy	1	43.343	<0.001
	family	4	257.607	<0.001
	family: species	9	45.83	<0.001

When comparing the MTG for the untreated (control) seed lot, the tested species in the Cistaceae, Cyperaceae and Urticaceae needed significantly more time to germinate than species of the Fabaceae and Poaceae (Appendix, Table A 2.2). Furthermore, germinability differed between plant families with low germination percentages for species in the Cistaceae, while most seeds germinated in the Fabaceae and Poaceae (Table 2.2). However, within several plant families germinability differed significantly between tested species, while no clear correlation was found between life strategy and germinability. No significant differences were found between both simulated herbivores (cattle and horse) for the mechanical (MC and MH), thermal (T49 and T55) and combination treatments (combiC and combiH) (Table 2.2).

No significant differences were found between treatments in the Cistaceae for MTG, while the mechanical treatment using sandpaper resulted in higher germinability for both *Cistus albidus* and *Helianthemum nummularium* (Table 2.2). Furthermore, significantly fewer seeds germinated following both heat treatments in *C. albidus*. None of the treatments had a significant effect on the Cyperaceae species. Thermal treatments had an adverse effect on the germinability of the *Trifolium* species and *Medicago lupulina* (T72). The chemical treatment HCl-P60 resulted in lower germinability for *Trifolium repens* while both combination treatments resulted in lower germinability for all *Trifolium* species. All treatments except MH resulted in a higher germinability compared to the control treatment for *Urtica urens*, while no significant differences were found between the treatments for *Urtica dioica* (Table 2.2).

Table 2.2 - Average values and standard errors for germinability (%) by treatment and species. Different letters on the same row indicate significant differences between treatments within one species (one-way ANOVA and Tukey HSD tests). Different letters in the species column indicate either significant differences between species within each plant family (lower-case letters) or plant families (upper-case letters) for the untreated seed lot. Annual species are underlined. Bold values in the treatment columns indicate significant differences from the control seed lot.

species	control	MS	MC	MH	HCl
Cistaceae **A**					
Cistus albidus **a**	23.2±3.0a	**34.8±3.5b**	24.4±1.5ab		26.0±1.3ab
Helianthemum nummularium **ab**	13.3±2.4a	**24.0±3.3b**	5.6±1.7a	6.9±0.7a	7.8±1.0a
Tuberaria guttata **b**	9.0±3.7ab		8.0±3.5ab	3.5±0.7a	20.7±3.5b
Cyperaceae **BC**					
Carex acuta **a**	42.0±2.4a	44.0±3.6a	40.0±3.6a		39.2±3.8a
Carex flacca **b**	70.8±10.3a	83.2±2.7a	87.2±3.3a		82.0±2.8a
Fabaceae **BD**					
Medicago arabica **a**	13.6±2.6a	**81.2±5.1b**			16.8±2.6a
Medicago lupulina **b**	89.2±1.5ab	91.2±1.7a	93.6±1.8a		92.4±1.7a
Trifolium arvense **b**	83.8±1.9ab	90.8±1.4a	82.3±2.2ab	77.1±0.0b	80.3±2.4b
Trifolium campestre **c**	38.2±2.5a		27.4±2.4ab	26.0±5.0ab	39.3±4.7a
Trifolium pratense **d**	60.4±5.5a		52.0±4.2ab	50.7±2.5ab	61.3±1.8a
Trifolium repens **b**	86.5±1.1a	86.8±3.7a	83.4±2.2ab	90.7±1.3a	81.3±2.8ab
Poaceae **D**					
Poa annua **a**	96.2±0.8ab	97.6±0.7a	97.5±0.7a	99.3±0.7a	97.5±0.9a
Poa pratensis **b**	62.4±3.7ab	63.6±2.7ab	67.8±1.5ab	75.3±1.8ab	67.0±2.3ab
Urticaceae **AC**					
Urtica dioica **a**	52.1±3.6a		52.4±10.9a	63.5±10.0a	63.5±8.5a
Urtica urens b	26.0±6.0a		**54.0±2.3bc**	50.7±5.4ab	**76.9±4.6d**

HCl-P15	HCl-P60	T24	T49	T55	T72	combiC	combiH
	24.0±3.2a	**6.4±1.7c**			**3.2±0.5c**		
10.7±5.7a	5.2±1.7a	3.6±1.3a	5.3±1.3a	7.0±2.1a	4.0±1.4a	6.1±3.1a	6.0±3.1a
7.3±3.7ab			0.7±0.7a	5.3±3.5a		6.0±4.2ab	7.4±1.3ab
	29.6±4.3a	43.7±4.2a			43.6±5.8a		
	89.6±3.0a	71.9±10.0a			88.4±2.0a		
	28.0±5.3a	14.4±1.6a			18.7±3.4a		
	89.6±3a	74.8±3.9b			**0.8±0.5c**		
78.0±5.3b	83.2±1.0ab	**52.0±1.4c**	**3.3±1.8d**	**16.0±15.0d**	**3.6±1.7d**	**3.3±1.3d**	**1.3±0.7d**
44.7±3.3a			**7.3±2.4b**	**13.3±4.1b**		**13.3±1.3b**	**12.0±6.9b**
69.3±13.8a			**5.3±2.4c**	**12.7±2.4bc**		**13.3±1.8bc**	**3.3±1.3c**
78.7±7.4ab	**73.6±2.6b**	**50.0±2.5c**	**38.0±6.4cd**	**33.3±0.7cd**	**1.6±0.7e**	**37.3±5.8cd**	**15.3±2.9de**
98.0±1.2a	97.6±1.6a	95.2±1.0ab	97.3±1.8a	92.0±1.2ab	86.0±2.1b	96.7±1.8ab	95.3±2.4ab
65.3±2.4ab	76.4±5.6a	72.8±3.8ab	55.3±6.8b	71.3±5.8ab	55.6±2.6b	71.3±4.1ab	54.0±9.5b
46.9±5.9a			42.7±1.8a	26.9±7.6a		40.3±9.2a	48.7±4.8a
58.0±5.2bcd			**74.5±4.7cd**	**64.7±1.8bcd**		**57.7±1.4bcd**	**75.3±4.7d**

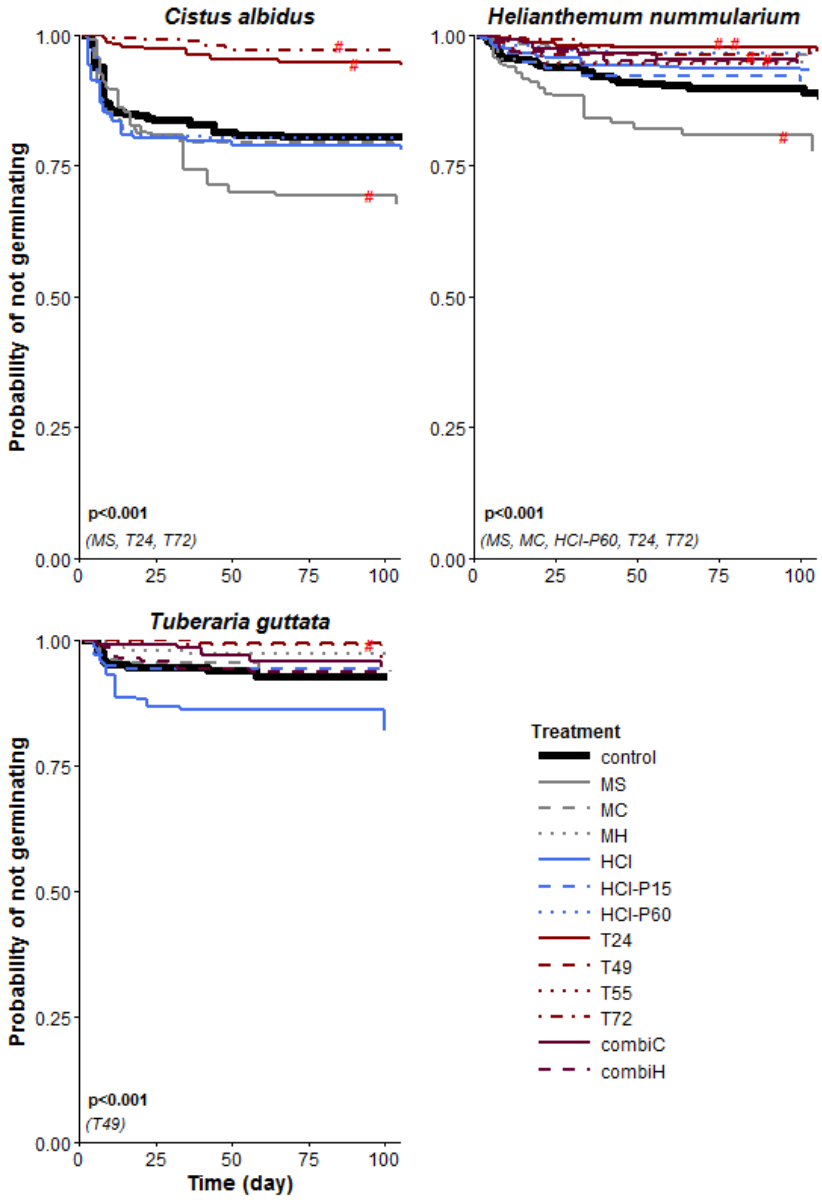


Figure 2.1 - Probability of not germinating (Kaplan-Meier estimates) following simulated gut treatments. Treatment curves which differ significantly from the control treatment after applying AFT-models are indicated with # and are listed in the graphs.

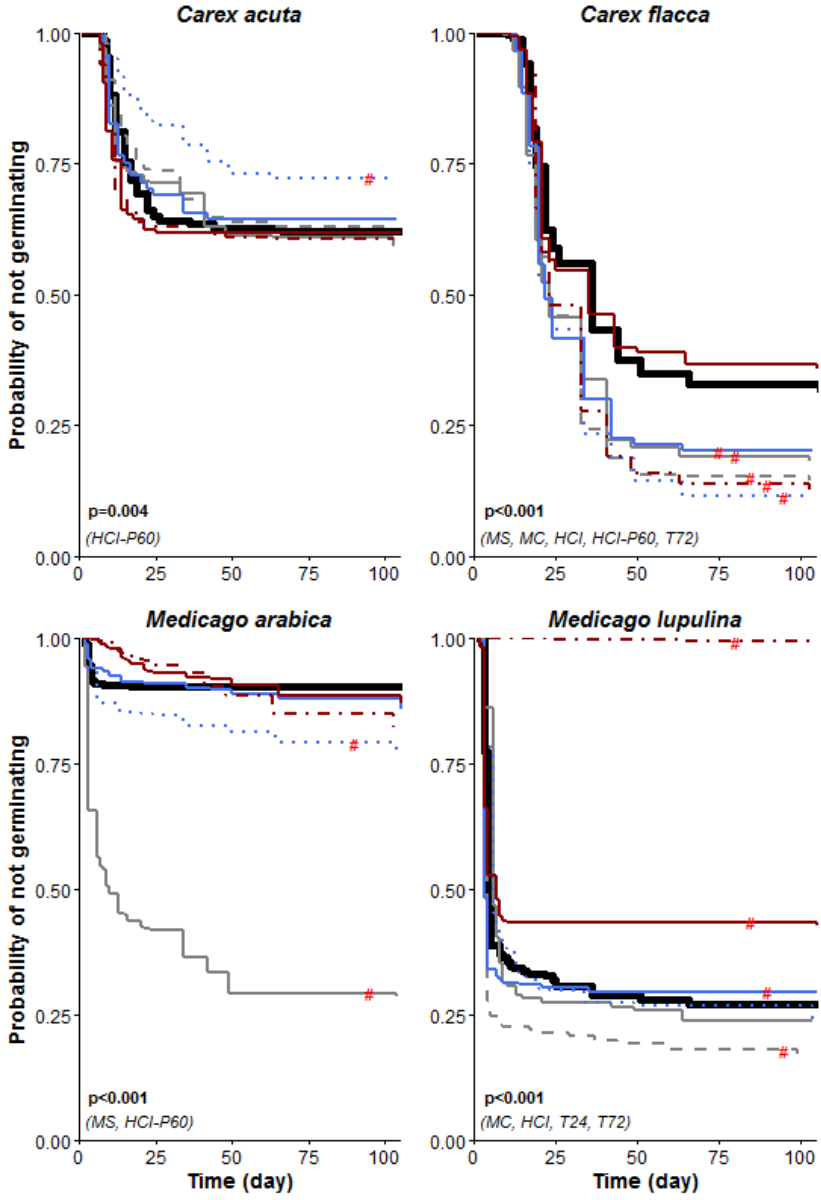


Figure 2.1 continued.

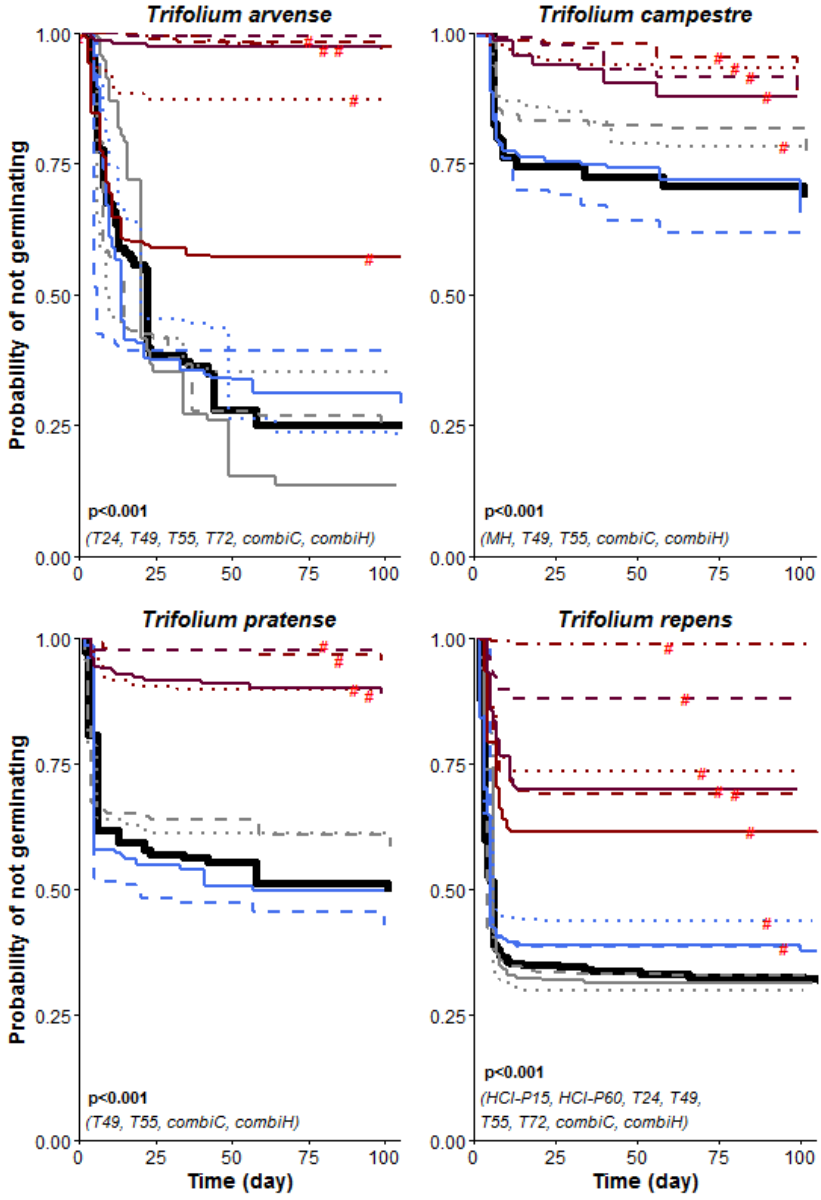


Figure 2.1 continued.

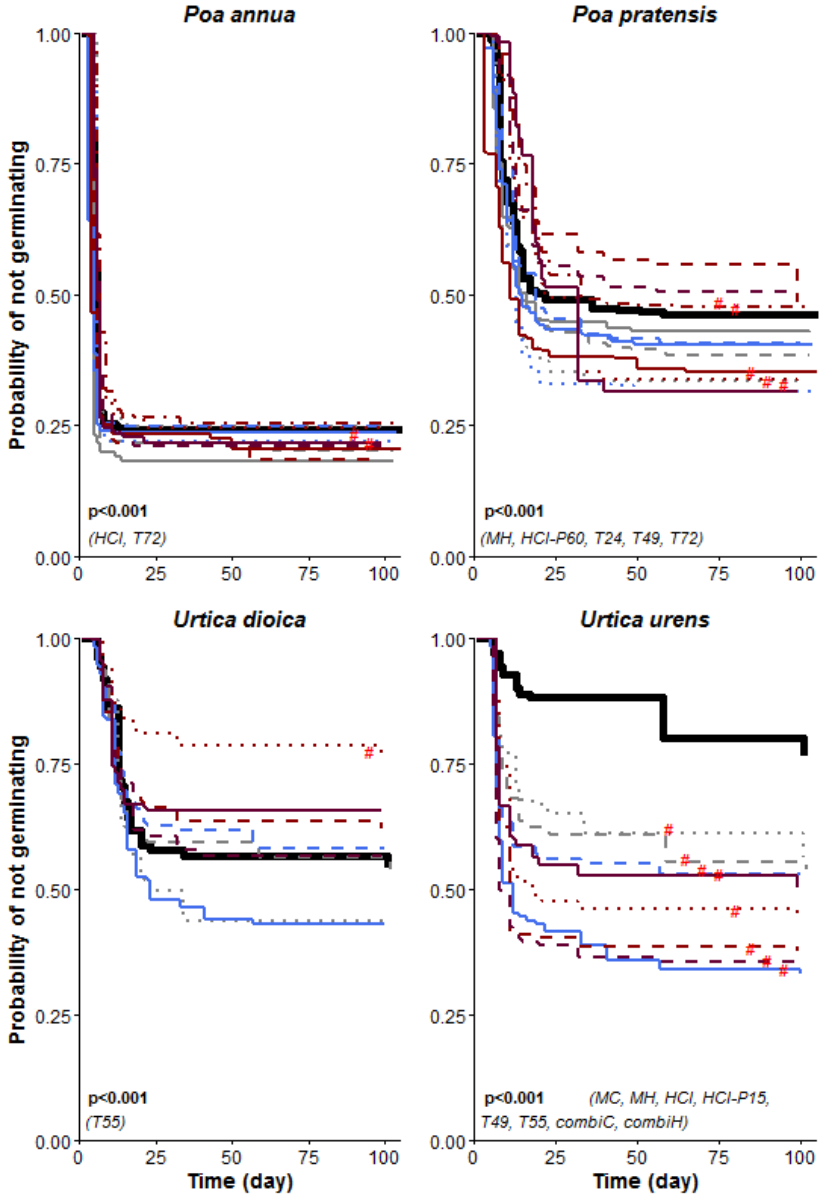


Figure 2.1 continued.

Survival analysis

Figure 2.1 compares the germination probabilities of the treatments with the control treatment. These survival curves depict the germinability and time to germination which differed greatly between species. For most of the tested species the survival curves of the treated seeds differ significantly from those of the untreated seed lots. Cistaceae species were mainly affected by mechanical and thermal treatments and to a lesser extent by chemical treatments, thermal treatments significantly altered the germination curves of all Fabaceae species except *Medicago arabica*. All applied treatments had a significant effect on the germination probability of *Urtica urens*, while the congeneric species *U. dioica* was only affected by the T55 treatment (Figure 2.1).

The time ratio which compares the germination times of the treated seeds with those of the reference seed lot (control treatment) for each species indicates a decelerating germination following the thermal and combination treatments for the tested Fabaceae species except *M. arabica* (Appendix, Table A 2.3). A clear difference was found for *U. urens* where any treatment accelerated germination. Chemical treatments had mainly an accelerating effect on *Carex flacca* and both *Poa* species.

Discussion

Most of the treatments had a decelerating and inhibiting effect on germination compared to the untreated seeds for most tested species. Species of the Cyperaceae and Poaceae were hardly impaired by any of the treatments and even germinated better after chemical treatments. Thermal treatments, simulating the body temperature, inhibited germination in most cases. The germination success of *Urtica urens* was significantly higher under all treatments, while species of the Cistaceae and Fabaceae particularly benefitted from mechanical treatments. Nevertheless, accelerated germination might be beneficial, especially in competitive environments (Orrock and Christopher, 2010). Dung pats can contain high numbers of endozoochorically dispersed seeds (e.g., Cosyns et al. (2005a)), and can therefore be regarded as highly competitive environments where

early germinating individuals can benefit from the vegetation-free and nutrient-rich environment of the dung pat (Verdú and Traveset, 2005).

The endozoochorous dispersal potential differs between species and depends on plant traits such as diaspore release height, and the morphology of the ingested diaspores. Survival after endozoochory has been especially correlated with persistent, small, rounded diaspores with a hard seed coat (Albert et al., 2015b). Hardseededness, a specific type of physical seed dormancy, is a widespread trait in flowering plants and is characterized by the prevalence of a water impermeable seed coat. Cistaceae species have a high incidence of this type of dormancy and do not germinate easily (Thanos et al., 1992). Mechanical scarification of the hard seed coat is a well-known practice to induce germination, along with thermal pre-treatment of the seeds (Delgado et al., 2001; Delgado et al., 2008; Pérez-García and González-Benito, 2006; Tavşanoğlu and Çatav, 2012). But even if the small and hard seeds of Cistaceae are softened by fire or scarification, the germination rate often remains low. Nevertheless, species with dormant seeds are able to escape from crowding or sibling competition by spreading their germination over several seasons (Russi et al., 1992). This is especially a benefit in the summer-dry and fire-prone Mediterranean climate zone which coincides with the distribution of most Cistaceae species (Thanos et al., 1992). Another link has been made between physical dormancy and animal mediated dispersal. When seeds are ingested by herbivores water-impermeable seeds are more likely to survive the digestive tract (Gardener et al., 1993b) and it has been pointed out that in an evolutionary context, dormancy increases with increasing dispersal while seed size decreases (Venable and Brown, 1988). In our experiment, the mechanical scarification of the seed coat of *C. albidus* and *H. nummularium* increased germinability and germination rate, which is in line with the findings of Pérez-García et al. (2006). Thermal treatments and chemical treatments using sulphuric acid were also applied by Pérez-García et al. (2006), but didn't lead to conclusive results although there was a general trend towards lower germination following these treatments, which is supported by our results for all tested Cistaceae species. The poor response of these species to our thermal treatments is remarkable as species in this family are known to benefit from high temperatures and even fire (Delgado et al., 2001; Delgado et al., 2008; Tavşanoğlu and Çatav, 2012). In Germany, the germination of *H. nummularium,* which under natural conditions usually takes place in

autumn, was even accelerated towards spring after a controlled fire experiment during spring (Poschlod et al., 2011). On the other hand, our thermal treatments simulating the wet and warm environment in herbivore guts can hardly be compared with the hot and dry environment characteristic for fires, and the temperatures were presumably not high enough to damage the seed coat. We should also keep in mind that the experimental period of 100 days might be rather short for slow germinating species. A viability test on the ungerminated seeds would have allowed us to differentiate between dead and dormant seeds.

Another family characterized by physical dormancy caused by a water impermeable seed coat are the Fabaceae. Gresta et al. (2007) even concluded that mature seeds of *Medicago arabica* and *Medicago lupulina* always have impermeable seed coats. The germinating seeds in our control treatment might indicate that physical dormancy was not yet established in some of the *Medicago* seeds. Physical dormancy also occurs in *Trifolium* species. In a germination experiment using seeds of wild populations of *Trifolium repens*, up to 35% of all viable seeds proved to be water-impermeable at maturity although this is mainly determined by the moisture conditions during seed maturation (D'hondt et al., 2010). In our experiment, the germination of *M. arabica* was stimulated after mechanical scarification with sandpaper, while this treatment had no significant effect on any other species in the legume family. The chemical, thermal and combination treatments rather had a negative effect on germination which suggests that gut passage results in viable seed loss. Similar experiments simulating digestion by sheep (Peco et al., 2006a) or cattle (Gardener et al., 1993b) indeed found a lower germinability of Mediterranean grassland species and higher survival percentage of hard-seeded legumes compared to soft-seeded species. In our experiment, the duration of the thermal treatment and germinability of legume species were negatively correlated. However, other factors may be causing these results. After 10-14 days, the major part of the ungerminated legume seeds started to show symptoms of fungal and bacterial infections characterized by green, black or white hyphae or pink slime, whereas few seeds of other species were infected. These infections were most probably initiated during the thermal treatment after which most of the seeds were clearly swollen. Imbibition is the first step in seed germination, but it also makes the embryo and cotyledons susceptible to pathogens (Baskin and Baskin, 2001).

Nevertheless, many Cistaceae and Fabaceae species were found in dung and their germination often was proved to be higher after gut passage. Goat feeding experiments resulted in the retrieval of high numbers of *Cistus albidus* seeds (Grande et al., 2013) and even increased germination of *Cistus salvifolius* (Mancilla-Leytón et al., 2011). Furthermore, the germination of six Mediterranean Cistaceae species increased up to seven-fold after a feeding experiment with sheep (Ramos et al., 2006). On the other hand, in a feeding experiment using sheep, cattle, rabbits, horses and donkeys the germination success was lower for *Helianthemum nummularium*, *Trifolium arvense*, *Trifolium campestre*, *Trifolium pratense* and *Trifolium repens* (Cosyns et al., 2005b), whereas a similar experiment using cattle (D'hondt et al., 2011) achieved higher germinability of *H. nummularium* and *T. pratense* seeds. *Urtica dioica* is known to germinate abundantly from dung (Cosyns et al., 2005a; Cosyns and Hoffmann, 2005; Couvreur et al., 2005a; Pakeman et al., 2002) and, in our experiments, germination was not impaired by any of the treatments, indicating that this species indeed is well-adapted to endozoochorical dispersal. More interestingly, treated seeds of the congeneric species *Urtica urens* germinated significantly better and faster compared to the control seed lot, which suggests that this species is able to benefit twice from endozoochory: once by the dispersal movement itself and additionally by an increased germination success at the deposition site. However, despite this high germination success *U. urens* has been rarely found germinating in dung and when found, few individuals emerge from dung (Malo and Suárez, 1996).

Our treatments had a very limited effect on the germinability of the tested *Carex* and *Poa* species, which conflicts with other studies where germination was negatively affected by gut passage (Cosyns et al., 2005b; D'hondt et al., 2011). The time ratio even indicates that germination of *Poa pratensis* and *Carex flacca* is accelerated after chemical treatment with hydrochloric acid, which is also supported by the results of Salehi and Khosh-Khui (2005) who achieved a higher germinability and germination rate of *P. pratensis* after bathing the seeds in sulphuric acid. Diaspore morphology might explain the observed germination patterns, as achenes (*Carex* species) and caryopses containing lemma and palea (*Poa* species) were used instead of seeds. These structures might have protected the seed from our gut simulation treatments. Furthermore, while it is commonly known that these species are successfully dispersed by endozoochory (e.g., Cosyns et al. (2006), Cosyns et

al. (2005a), Couvreur et al. (2005a), Pakeman et al. (2002)), we should keep in mind that we as well as Salehi and Khosh-Khui (2005) did not simulate all steps of the digestive tract, while the other studies used true gut passage to test germination effect. We simulated the effects of the acid environment in the stomach and duodenum and the effect of a proteolytic enzyme (pepsin) in combination with an acid, whereas the amylolytic and lipolytic enzymes produced in the digestive system and the proteolytic and cellulolytic enzymes secreted by bacteria were left out in this experiment. In addition to the effects of mechanical, thermal and chemical scarification in the gastrointestinal environment, seed coat abrasion due to microbial activity might, therefore, provide us further insight in the survival probability of seeds during endozoochory. Although the endozoochorous dispersal of plant seeds is correlated with diaspore size, shape, seed coat morphology, seed longevity and seed production, ungulate traits (e.g., habitat use, diet and digestive system) are at least as important in determining whether a plant species is an endozoochorous disperser (Albert et al., 2015b).

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Appendix

Table A 2.1 - Plant species and families used in the experiment, life strategies (with A= annual and P= perennial), seed origin (with C= Cruydt Hoeck, http://www.cruydthoeck.nl/ and S= Semillas Cantueso, http://www.semillascantueso.com/) and applied experimental treatments (with MS= sandpaper treatment, MC= cattle mastication treatment, MH= horse mastication treatment, HCl= hydrochloric acid treatment, HCl-P15= hydrochloric acid+peptide treatment during 15 minutes, HCl-P60= hydrochloric acid+peptide treatment during 60 minutes, T24= thermal treatment during 24h, T49= thermal treatment during 49h, T55= thermal treatment during 55h, T72= thermal treatment during 72h, combiC= combination of treatments MC, HCl-P15 and T49, and combiH= combination of treatments MH, HCl-P15 and T55).

plant family and species	life strategy	seed origin	control	MS	MC	MH	HCl	HCl-P15	HCl-P60	T24	T49	T55	T72	combiC	combiH
Cistaceae															
Cistus albidus	P	S	x	x	x		x		x	x			x		
Helianthemum nummularium	P	C	x	x	x	x	x	x	x	x	x	x	x	x	x
Tuberaria guttata	A	S	x		x	x	x	x			x	x		x	x
Cyperaceae															
Carex acuta	P	C	x	x	x		x		x	x			x		
Carex flacca	P	C	x	x	x		x		x	x			x		
Fabaceae															
Medicago arabica	A	C	x	x			x		x	x			x		
Medicago lupulina	P	C	x	x	x		x		x	x			x		
Trifolium arvense	A	C	x	x	x	x	x	x	x	x	x	x	x	x	x
Trifolium campestre	A	C	x		x	x	x	x			x	x		x	x
Trifolium pratense	P	C	x		x	x	x	x			x	x		x	x
Trifolium repens	P	C	x	x	x	x	x	x	x	x	x	x	x	x	x
Poaceae															
Poa annua	A	C	x	x	x	x	x	x	x	x	x	x	x	x	x
Poa pratensis	P	C	x	x	x	x	x	x	x	x	x	x	x	x	x
Urticaceae															
Urtica dioica	P	C	x		x	x	x	x			x	x		x	x
Urtica urens	A	C	x		x	x	x	x			x	x		x	x

Table A 2.2 - Average values and standard errors for mean time to germination (MTG, day) by treatment and species. Different letters on the same row indicate significant differences between treatments within one species (one-way ANOVA and Tukey HSD tests). Different letters in the species column indicate either significant differences between species within each plant family (lower-case letters) or plant families (upper-case letters) for the untreated seed lot. Annual species are underlined. Bold values in the treatment columns indicate significant differences from the control seed lot.

Species	**control**	**MS**	**MC**	**MH**	**HCl**
Cistaceae **A**					
Cistus albidus **a**	13.9±2.2ab	23.8±4.5ab	10.7±1.7ab		12.1±2.2ab
Helianthemum nummularium **a**	30.9±5.4a	30.8±6.0a	29.8±8.5a	7.2±1.4a	18.5±4.2a
Tuberaria guttata **a**	26.0±9.9a		47.1±28.3a	18.7±7.7a	26.5±6.6a
Cyperaceae **A**					
Carex acuta **a**	16.3±2.0ab	20.9±4.4ab	20.5±3.7ab		15.7±2.4ab
Carex flacca **b**	28.7±3.6a	25.7±1.2a	25.8±1.2a		24.5±1.3a
Fabaceae **B**					
Medicago arabica **a**	5.6±2.2a	10.8±2.8ab			22.7±7.6abc
Medicago lupulina **a**	6.1±0.5abc	7.0±0.4ab	4.9±0.6bc		3.6±0.2c
Trifolium arvense **b**	16.0±2.6abcd	22.2±2.1abc	14.6±2.9bcd	10.1±1.9bcd	13.0±2.0bcd
Trifolium campestre **b**	15.7±2.2a		22.7±9.6a	18.8±5.7a	23.2±5.9a
Trifolium pratense **ab**	11.2±1.6ab		11.0±1.8ab	4.8±0.5b	7.9±1.3ab
Trifolium repens **a**	5.1±0.4ab	5.3±0.3ab	4.2±0.2a	5.4±0.1ab	4.4±0.8a
Poaceae **B**					
Poa annua **a**	5.3±0.3a	5.2±0.1ab	**6.0±0.3bc**	**6.2±0.1bc**	**4.3±0.3b**
Poa pratensis **b**	11.7±0.6a	11.1±0.7a	12.1±1.2a	10.8±0.4a	11.1±0.7a
Urticaceae **A**					
Urtica dioica **a**	16.4±3.8a		18.9±2.5a	14.9±1.2a	14.0±1.9a
Urtica urens **a**	31.7±10.0a		16.3±2.9ab	20.3±4.8ab	11.8±3.2ab

HCl-P15	HCl-P60	T24	T49	T55	T72	combiC	combiH
	10.0±2.7b	34.7±11.1a			28.9±7.6ab		
29.2±12.3a	17.0±8.8a	32.5±11.6a	25.3±13.3a	13.2±1.2a	45.0±15.0a	33.3±5.8a	16.9±3.8a
7.2±0.2a			40.0a	57.5±41.5a		41.8±11.3a	20.0±6.5a
	24.2±4.6a	11.5±0.7b			12.7±0.8ab		
	26.2±0.8a	26.3±1a			27.9±0.9a		
	19.6±8.1abc	**38.6±9.9bc**			**42.5±5.4c**		
	8.1±0.7a	4.5±0.6bc			**43.0±20.0d**		
5.5±0.2d	21.6±6.4abcd	8.6±0.7bcd	55.9±9.6a	6.2±2.2cd	42.8±10.8ab	10.9±5.6bcd	56.5±42.5ab
15.2±3.5a			55.4±25.3a	44.5±21.7a		28.3±4.7a	49.0±7.3a
10.3±2.8ab			35.6±14.9a	8.4±1.7ab		21.1±2.4a	4.0±0.0b
5.3±0.1ab	4.0±0.3a	6.5±0.9ab	6.7±0.5ab	6.3±0.4ab	8.5±2.0b	6.9±0.4ab	5.9±0.4ab
5.2±0.0ab	5.7±0.2a	5.5±0.5a	6.3±0.3ac	6.4±0.1ac	**7.4±0.4c**	6.2±0.2ac	6.6±0.1ac
13.6±0.5ab	11.4±0.3a	9.2±1.2a	**26.9±7.4c**	**19.0±0.9bc**	**17.6±0.6bc**	**20.4±0.4bc**	14.4±1.9ab
15.6±0.9a			15.3±2.0a	22.7±10.0a		10.7±0.1a	14.0±1.2a
9.8±1.2ab			10.1±0.9ab	12.2±0.9ab		12.7±1.9ab	**8.9±0.6b**

Table A 2.3 - Time ratio (TR) and standard errors by species and treatment after applying AFT models. TR>1 indicates decelerated germination and TR<1 indicates accelerated germination compared to the control treatment for a particular species. Asterisks indicate significance levels with ***: $p<0.001$, **: $0.001<p<0.010$ and *: $0.010<p<0.050$. Annual species are underlined.

species	control	MS	MC	MH	HCl
Cistaceae					
Cistus albidus	1	0.5±0.2*	0.8±0.3		0.7±0.2
Helianthemum nummularium	1	0.3±0.1*	4.2±2.1**	2.3±1.5	2.1±1
Tuberaria guttata	1		1.2±1.3	3.7±4.1	0.2±0.2
Cyperaceae					
Carex acuta	1	0.9±0.3	1.2±0.3		1.1±0.3
Carex flacca	1	0.7±0.1*	0.6±0.1*		0.7±0.1*
Fabaceae					
Medicago arabica	1	<0.1±0.1***			0.7±0.3
Medicago lupulina	1	1.2±0.1	0.7±0.1***		0.8±0.1*
Trifolium arvense	1	1.1±0.3	0.9±0.2	0.9±0.3	1.0±0.2
Trifolium campestre	1		2.2±1.0	2.4±1.0*	1.0±0.4
Trifolium pratense	1		1.4±0.6	1.4±0.6	0.9±0.4
Trifolium repens	1	1.1±0.2	1.0±0.2	1.1±0.2	1.2±0.2
Poaceae					
Poa annua	1	0.9±0.1	1.1±0.1	1.2±0.1	0.8±0.1**
Poa pratensis	1	0.8±0.2	0.8±0.1	0.6±0.1*	0.8±0.1
Urticaceae					
Urtica dioica	1		1.1±0.5	0.7±0.3	0.6±0.3
Urtica urens	1		0.2±0.1***	0.3±0.1***	0.1±0.0***

HCl-P15	HCl-P60	T24	T49	T55	T72	combiC	combiH
	0.8±0.3	13.2±6.1***		36.2±20.2***			
1.4±0.9	4.3±2.5**	8.1±5***	3.8±2.6	2.7±1.8	7.4±4.5***	3.7±2.5	3.3±2.2
1.3±1.4			32.1±47.6*	3.3±3.6		2.5±2.7	1.2±1.3
	2.1±0.6**	0.8±0.2			0.8±0.2		
	0.6±0.1**	0.9±0.2			0.7±0.1*		
	0.3±0.1**	1.0±0.4			0.7±0.3		
	1.2±0.1	1.5±0.2**			772.6±476.8***		
0.7±0.2	1.2±0.3	1.8±0.5*	49.1±19.9***	13.9±4.9***	49.5±16.3***	36.5±14.0***	79.1±37.3***
0.6±0.3			21.7±11.3***	8.9±4.2***		8.1±3.8***	11.5±5.6***
0.7±0.3			118.9±70.6***	28.1±14.4***	27.7±14.2***	164.7±103.6***	
1.6±0.3*	1.6±0.3**	5.3±0.9***	9.2±2.0***	11.8±2.6***	502.5±191.3***	9.7±2.1***	40.8±10.4***
1.0±0.1	1.0±0.1	0.9±0.1	1.1±0.1	1.2±0.1	1.4±0.1***	1.1±0.1	1.2±0.1
0.9±0.2	0.6±0.1**	0.5±0.1***	1.6±0.3*	0.9±0.2	1.5±0.3*	1±0.2	1.4±0.3
1.2±0.5			1.4±0.6	3.3±1.5**		1.5±0.6	1.1±0.5
0.2±0.1***			0.1±0.0***	0.1±0.0***		0.2±0.1***	0.1±0.0***

3 Reduced germination success of temperate grassland seeds sown in dung: consequences for post-dispersal seed fate

Tanja Milotić and Maurice Hoffmann

Planting pots in the outdoor experiment (Wenduine, Belgium).

Modified from Milotić, T. and Hoffmann, M. (2016) Reduced germination success of temperate grassland seeds sown in dung: consequences for post-dispersal seed fate. Plant Biology, **18**(6), 1038-1047, DOI: 10.1111/plb.12506

Abstract

Endozoochory is one of the main drivers shaping temperate grassland communities by maintaining plant populations and enabling plants to colonize new habitats. Successful endozoochorous dispersal implies that seeds not only get consumed and survive the digestive tract but are also able to develop into viable seedlings in a dung environment.

We experimentally assessed the germination probability and timing of 15 annual and perennial temperate European grassland species in cattle and horse dung and in different climatic conditions (greenhouse and outdoor conditions).

Interspecific variation in germinability and germination timing are found, while life strategy had only an effect on germination timing. We found adverse effects of both cattle and horse dung on the germination characteristics of all tested grassland species, but the effects of cattle dung were more pronounced. In comparison with the control treatment, fewer seeds emerged in dung and more time was needed to germinate. Also, germination metrics clearly differed between the artificial greenhouse and outdoor conditions, with generally a lower germinability in outdoor conditions.

According to our results, a large cost seems to be associated with endozoochorous dispersal in this stage of the life cycle, as seed dispersal effectiveness strongly depends on the quality of the deposition site with a lowered survival and germination probability when seeds are deposited in dung.

Keywords: dispersal success, endozoochory, germination, grassland species, seed dispersal

Introduction

Seed dispersal has a direct influence on individual plant fitness as the deposition site determines whether a seed will live, and eventually germinate and establish, or die (Wenny, 2001). Consequently, plant community composition, structure and dynamics are initially shaped by the spatial patterns of seed dispersal and dispersal success (Nathan and Muller-Landau, 2000; Schupp and Fuentes, 1995). Diaspore morphology determines to a large extent whether the most likely dispersal vector is abiotic (e.g., wind, water) or biotic (animals or the plant itself) (Albert et al., 2015b; Levin et al., 2003). One of the animal-mediated dispersal processes is endozoochory implying that seeds get dispersed via ingestion by vertebrate animals and consecutive deposition in a dung environment. Endozoochory has been documented in a wide range of habitats and latitudes from species carrying fleshy, palatable fruits (Traveset et al., 2007) to herbaceous species lacking any apparent morphological adaptations to dispersal (Mouissie et al., 2005c; Pakeman et al., 1998). The 'foliage is the fruit' hypothesis postulates that grazing herbivores are attracted to the palatable foliage of the species in the latter group and ingest seeds rather inadvertently (Janzen, 1984). Endozoochory by large herbivores enables long distance seed dispersal as the gut retention time of seeds is rather long (Cosyns et al., 2005b) and animal mobility can be high (Nathan et al., 2008), and is often much higher than other dispersal agents. Furthermore, many germinable grassland species have been found highly concentrated in herbivore dung which suggests that endozoochory is one of the main drivers, shaping temperate grassland communities (Cosyns et al., 2005a; Couvreur et al., 2005a; Pakeman et al., 1998).

In recent decades, zoochorous dispersal of grassland seeds and the consequences for nature restoration and conservation gained much scientific attention. Nevertheless, most of these studies focus on diet choice, animal behaviour and/or the presence of germinable seeds in dung, and, therefore, provide valid proof for the existence and relevance of this dispersal mode, but few examine the consequences of the quality of depostion sites. Undoubtedly, the first step in successful endozoochorous dispersal includes seed survival in the consecutive digestive processes (mastication, rumination and digestion). Gut passage may result in the breaking of dormancy and thus in enhancing or reducing germination

success and timing (D'hondt and Hoffmann, 2011; Traveset, 1998). Besides that, the deposition of seeds in a highly nutritive environment such as dung may affect germination and the early development of seedlings as well (Deshaies et al., 2009).

The effect of dung on post-dispersal germination and growth is less studied and is subject to rather contrasting results. Fresh dung often suppresses the existing vegetation and creates, therefore, a beneficial microhabitat for germination by eliminating competition with the already developed vegetation (Traveset, 1998). Dung can also shape plant communities by changing the relative abundance of species in the soil seed bank and by providing extra nutrients for the growth and flowering phase of endozoochorously dispersed seeds (Dai, 2000; Traveset, 1998). The moist and nutrient-rich dung environment leads in some cases to more and faster germination (e.g., Archer and Pyke (1991), Carmona et al. (2013), Malo and Suárez (1995a), Quinn et al. (1994), Traveset et al. (2001)), while germination was inhibited in other studies (e.g., Carmona et al. (2013), Izhaki and Ne'eman (1997), Paré et al. (1997)). Besides a high concentration of growth promoting macronutrients (N, K and to a lower extent P, Ca and Mg) and essential trace minerals (e.g., Fe, Mn and Cu) (Haynes and Williams, 1993; Lupwayi et al., 2000), animal excrements contain growth-inhibiting substances such as phenolic compounds and fatty acids. These phytotoxic compounds may alter the activity of enzymes that regulate the germination rate and, therefore, inhibit germination of certain plant species (Marambe et al., 1993).

The chemical and structural composition of fresh dung highly depends on herbivore diet (e.g., captive versus free-ranging animals), but dung quality is also linked with the nutritive physiology of mammals as on average dung from non-ruminant species is more fibrous and has a lower nitrogen content compared with ruminant dung (Holter, 2016). Furthermore, the composition of dung changes while ageing, with decreasing water and nitrogen content (Holter, 1991; Holter, 2016), and the nature of these changes might also vary between dung types. The rapid desiccation of dung types consisting of small pellets (such as sheep and rabbit excrements) or the appearance of a hard and dry crust on watery dung types (such as cattle dung) may also prevent the imbibition of seeds prior to germination (Eichberg et al., 2007; Welch, 1985). Depending on the climatic circumstances and its composition, dung can become a very dry environment which is suboptimal for

germination and seedling growth. This is certainly the case with cow dung and dung of many other ruminants. Dung pad desiccation starts superficially creating a dry, more or less impermeable crust, isolating seeds in the dung from the local environment; in the end, the dung pat may dry out completely. Many germination trials have been conducted either in controlled greenhouse conditions or in outdoor conditions. In a greenhouse or standardized laboratory environment, the temperatures are likely to be higher and more constant and the water supply is kept optimal for plant growth, which makes it hard to extrapolate greenhouse results to true outdoor conditions. Studies comparing both greenhouse and natural conditions are rare, but generally found more and faster germination in controlled laboratory or greenhouse environments compared to outdoor conditions (Ramos-Font et al., 2015; Welch, 1985).

We conducted a dung addition experiment using 15 herbaceous plant species which are commonly found in temperate European grasslands and have a known potential for endozoochorous dispersal. Since cattle (used as a model species for a ruminant) and horses (used as a model species for a hindgut fermenter) are commonly introduced in nature management in these habitats, we used dung of both opposing herbivore types in the experiments. Environmental differences were addressed by replicating the experiment in both greenhouse and outdoor conditions. The questions raised in this study are:

1. What is the effect of the presence of dung on germinability and germination timing?
2. Why and to what extent do greenhouse conditions modify the germination process compared to outdoor or natural conditions?
3. Does the germination response differ between cattle (ruminant) and horse (hindgut fermenter) dung?

Materials and methods

Plant species

15 grassland species belonging to 7 plant families were used. In most families both annual and perennial species were selected as species capable of setting seeds the first year have a tendency to germinate sooner and more abundantly (Shipley and Parent, 1991). Most species are common in temperate European grasslands and were previously shown to germinate from cattle and horse dung (Cosyns et al., 2005a; Cosyns et al., 2005b; Cosyns and Hoffmann, 2005; Couvreur et al., 2005a). Cistaceae species are less common but were added as earlier research suggested that endozoochory might be an important dispersal mechanism in this family (Mancilla-Leytón et al., 2011; Manzano et al., 2005; Ramos et al., 2006). For example, the distribution of *Helianthemum nummularium* remarkably increased after the introduction of large herbivores in calcareous dune grasslands in Belgium and the germination rate of this species is known to increase after ingestion (D'hondt and Hoffmann, 2011; Provoost et al., 2015). *Tuberaria guttata* is a species with a Mediterranean distribution (Herrera, 2004) and has been found germinating in dung as well (Malo and Suárez, 1995b; Malo and Suárez, 1996). Intact seeds of Cistaceae species are known to germinate very slowly due to their hard and water-impermeable seed coat (Thanos et al., 1992) while mechanical scarification of seeds is commonly used to induce germination (Milotić and Hoffmann, 2016b; Pérez-García and González-Benito, 2006). In order to gain a complete overview of the germination process of the Cistaceae species, both intact and pre-treated seeds of *H. nummularium* and *T. guttata* were tested. Pre-treated seeds were mechanically scarified using fine grit sandpaper with average particle diameter of 125 µm. The scarification process was standardized by putting the seeds between two pieces of sandpaper which were moved twice.

Seed quality test

All seeds were purchased in specialized web shops. Prior to the dung addition experiments, seed quality was tested in standardized laboratory conditions. In this trial, 50 seeds per replicate were sown at equal distance

in Petri dishes filled with 1% water agar. Per species five replicates were used and all Petri dishes were arranged in a complete random design in a germination chamber with day: night fluctuating temperatures between 14°C and 30°C with a 12h day: 12h night period. Light was provided by white fluorescent tubes (Sylvania, 36W, Germany) with a light intensity (photosynthetic active radiation, PAR) between 10 and 45 µmol m^{-2} s^{-1} depending on the location on the rack. Newly germinated seeds were counted daily during the first 3 weeks of the trial, and afterwards once every 2 days until the 60-day germination trial period ended. Germination was considered to occur whenever a white radical emerged through the testa. After each counting round, all germinated seeds were discarded and the Petri dishes were put in new randomized sequence in order to lower the impact of possible unequal abiotic conditions in the germination chamber.

Dung addition experiment

The experiment was run simultaneously in outdoor and greenhouse conditions. At both locations, 3 treatments were used: cattle and horse dung treatments and a control treatment in which no dung was added. Round plastic plant pots (diameter: 15 cm, height: 16 cm) with drainage holes were either put on felt fabric in the greenhouse to limit evaporation or on a sturdy plastic screen in order to prevent interaction with the existing vegetation in the outdoor experiment. The outdoor experiment was covered with a net in order to prevent interference of wild rodents or birds with the experiment. Pots were filled with a 1:1 mixture of sand and compost. Dung was collected from stabled cattle and horses in order to keep contamination with wild seeds minimal. Before sowing, the pots were watered until the soil felt moist. 20 seeds were sown per pot with 20 replicates per treatment in both environments, summing to a total of 120 plant pots per species. In the treatments including dung, the entire surface of the pots was covered with dung and the seeds were mixed with dung in order to simulate seed deposition following endozoochory. In the control treatment, seeds were put homogeneously on the substrate surface in order to mimic dung-unassisted dispersal. Blank pots covered by either cattle or horse dung, or no dung (but without added seeds) were installed in both environments to check for seed contamination originating from the sand-compost mix, dung or surroundings. All pots were put in a complete randomized design. Pots in

the greenhouse were watered manually on a daily basis while outdoor pots were subjected to precipitation and did not receive any additional water. Both experiments were run in natural daylight cycles; no artificial lighting was used in the greenhouse. Seeds were sown on April 5th, 2012 at both locations. From then on, seed germination was monitored in 3-day intervals during the first 20 days of the experiment, while the counting effort was lowered to weekly counts afterwards. As the outdoor experiment got invaded by rabbits at day 65, the experiment was terminated at day 62 when the last rabbit-free count took place. In order to test the germination of slow-germinating species, the greenhouse experiment was monitored longer and was stopped at day 200 after no germination had taken place during the preceding 50 days. During the experiment, temperature and relative humidity of air and soil was measured in 15 min intervals using HOBO U23 Pro v2 Temperature/Relative Humidity Data Loggers (ONSET, USA). In total, four devices were used which either were put on top of a planting pot or dug into the substrate in outdoor and greenhouse conditions[2].

Data analysis

At the end of the experiments, germinability and mean time to germination (MTG) were calculated for each individual pot. Germinability was expressed as the cumulative percentage of seeds that had germinated by the end of the experiment while MTG was calculated as:

$$MTG = \frac{\sum_{i=1}^{k} t_i * n_i}{N}$$

where t_i is the time from the start of the experiment to the i^{th} observation, n_i is the number of seeds germinated within the period i, N is the total number of germinated seeds during the experiment and k is the last time of germination (Ranal and Garcia De Santana, 2006).

[2] According to the Belgian royal meteorological institute (KMI) **April 2012** was characterized by low average temperature (8.4°C versus the normal value of 9.8°C), high total precipitation (104.1 mm versus 51.3 mm) and normal sunshine duration (113:11 h). **May 2012** had normal values for the average temperature (14.3 °C), precipitation (63.4 mm) and sunshine (189:52 h) (source: www.meteo.be accessed January 2017).

All analyses were performed with R version 3.2.2 (R Core Team, 2015a). Germinability and MTG of the seeds in the seed quality test were compared among species using one-way ANOVAs and Tukey posthoc tests. Nested ANOVAs were used on the entire dataset of the dung addition experiment with germinability and MTG as measured variable and dung type, life strategy, environment and family as nominal variables with a nested family: species term. The initially included life strategy - dung and life strategy - environment terms were discarded from the final models as no significant interactions were found and all nominal variables were kept as main effects. For each individual species, the effects of cattle or horse dung addition and the environment of the pots (greenhouse or outdoors) on germinability were analyzed by two-way ANOVAs and Tukey posthoc tests in order to distinguish significantly differing dung types. In order to meet the assumptions of parametric tests, germinability and MTG data were respectively arcsine square-root and $\log_{10}$ transformed.

Survival analysis is increasingly being used to analyse germination data and is especially valuable in analysing germination timing in case germination is measured in time intervals (McNair et al., 2012; Onofri et al., 2010). The germination probability was estimated non-parametrically using the Kaplan-Meier method:

$$S(t) = \prod\nolimits_{j=1}^{s} \left(1 - \frac{d_j}{n_j}\right)$$

where d_j is the number of seeds germinating in a given interval of time j and n_j is the number of seeds "at risk" of germination in the same interval which is defined as the number of non-germinated seeds entering the interval minus one-half of the number of seeds germinated during that same interval (Venables and Ripley, 2002). Germination probability was calculated for each species and dung type using the function *survfit* in the package 'survival' version 2.38-3 (Therneau, 2015). The resulting germination curves were compared with AFT (accelerated failure time) regressions using the *survreg* function in R (Therneau, 2015). As suggested by Onofri et al. (2010) the problem of clustering seeds within randomization units (plant pots) was overcome by adopting a frailty approach with plant pots as the clustering (gamma distributed) random effect. Exponential, Weibull, Log-Normal and Log-Logistic distributions were tested per species. The best fitting model for each species was selected using the second order Akaike's information

criterion (AICc) and a graphical comparison of the model outcome with the Kaplan-Meier estimator. Time ratios were derived from the AFT-models using the method of Onofri et al. (2010) and imply that the germination timing in each dung type in both greenhouse and outdoor conditions is compared to the control treatment in greenhouse conditions. The control treatment has a fixed time ratio of 1. Treatments with a time ratio higher than 1 have longer germination times while time ratios smaller than 1 indicate accelerated germination compared to the control treatment.

Environmental conditions were expressed as cumulative growing degree-days (GDD) and the daily relative humidity which was calculated as the average value of all relative humidity measurements of each experimental day. GDD was calculated using the method recommended by McMaster and Wilhelm (1997):

$$GDD = \sum_{i=1}^{n} \left[\frac{\left(T_{max,i} - T_{min,i}\right)}{2} \right] - T_{base}$$

where $T_{max,i}$ and $T_{min,i}$ are respectively the maximum and minimum daily temperature recorded on day i, and T_{base} is set to a fixed 10°C which is an often used threshold value in temperate climates. If $[(T_{max,i} - T_{min,i})/2]$ is smaller than T_{base} then T_{base} equals $[(T_{max,i} - T_{min,i})/2]$ (McMaster and Wilhelm, 1997).

Results

Seed quality test

Interspecific variation in germinability and mean time to germination (MTG) was found (Table 3.1). The germinability of the *Trifolium* species, *Juncus effusus*, *Poa annua* and the scarified seeds of *Helianthemum nummularium* was near to 100% while very few seeds of both *Urtica* species germinated in the lab. MTG was high for *Stellaria media* and the Cistaceae species except the scarified seeds of *H. nummularium* while the *Trifolium* species had the fastest germination rate (Table 3.1).

Table 3.1 - Results of the seed quality tests of the seeds used in the dung addition experiments. Tests were performed in standardized laboratory conditions. Average germinability and mean time to germination (MTG) with standard errors are shown for the studied plant species and families with their respective life strategies. Different letters in the same column indicate significant differences between species after applying ANOVA and Tukey posthoc tests.

plant family and species	life strategy	germinability (%)	MTG (d)
Caryophyllaceae			
Stellaria media	annual	28.0±11.3 ABC	133.5±54.2 ab
Cistaceae			
Helianthemum nummularium	perennial		
intact seeds		10.4±2.5 CD	152.6±30.8 a
scarified seeds		90.4±2.0 E	24.2±7.2 bc
Tuberaria guttata	annual		
intact seeds		39.2±8.2 ABF	203.5±48.6 a
scarified seeds		44±4.3 ABF	83.3±27.1 ab
Fabaceae			
Trifolium pratense	perennial	88.8±2.6 E	1.9±0.2 de
Trifolium repens	perennial	97.6±0.4 E	1.5±0.0 e
Gentianaceae			
Centaurium erythraea	perennial	57.2±3.3 F	9.7±0.4 bcd
Juncaceae			
Juncus bufonius	annual	29.6±4.6 ABC	12.8±0.5 bc
Juncus effusus	perennial	90.8±2.5 E	10.9±0.8 bcd
Poaceae			
Agrostis capillaris	perennial	46.8±3.0 AF	22.7±8.6 bc
Agrostis stolonifera	perennial	22.0±3.6 ABC	10.1±1.8 bcde
Alopecurus myosuroides	annual	21.2±4.8 BC	44.6±14.3 ab
Poa annua	annual	95.6±1.0 E	4.6±1.3 cde
Poa pratensis	perennial	33.2±2.9 ABF	12.4±0.7 bc
Urticaceae			
Urtica dioica	perennial	2.0±0.6 D	22.1±8.3 bc
Urtica urens	annual	1.2±1.1 D	23.0±9.5 abc

Environmental conditions in the dung addition experiment

Environmental conditions clearly differed between the outdoor and greenhouse situation (Figure 3.1). Air measurements of GDD rose rapidly in the greenhouse at the start of the experiment but reached a steady level after 42 days once the difference between the minimum and maximum temperatures became minimal. In outdoor conditions, a higher cumulative

GDD was reached at the end of the experiment and the difference between air and soil GDD values was smaller than in greenhouse conditions. Relative humidity varied between 65 and 100%, except for the soil measurements in outdoor conditions where a nearly constant humidity of 100% was measured (Figure 3.1).

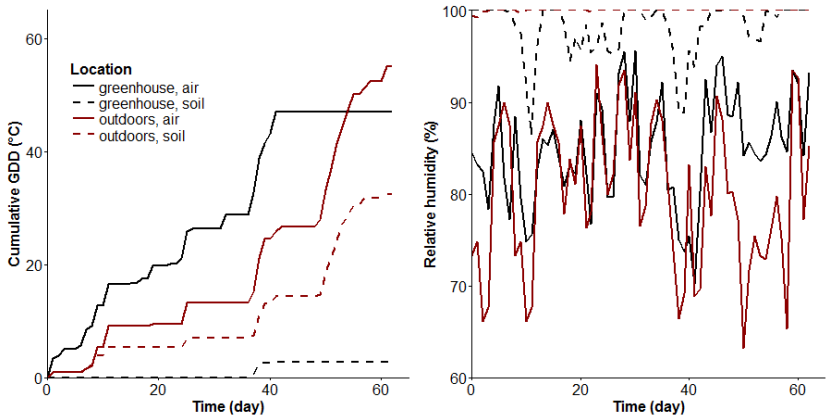

Figure 3.1 - Cumulative growing degree-day (GDD, °C) and relative humidity (%) of air and soil during the experiments in greenhouse and outdoor conditions.

Seed germination characteristics in dung environment

None of the selected test species emerged in the blank pots neither in cattle or horse dung or the sand-compost mix in the greenhouse and outdoor environment. Both germinability and MTG differed between species and families and were affected by dung type and environment (Table 3.2). Life strategy had only a significant impact on MTG, with annuals generally germinating faster than perennials. No seeds of *Urtica urens* germinated during the experimental period; therefore, this species was discarded from further analyses. The presence of dung decreased the germinability of seeds in greenhouse conditions, as significantly more seeds of all tested species germinated in the control pots compared to the pots where cattle or horse dung was added (Figure 3.2). In most cases, dung types did not affect germinability, but in the case where differences were found more seeds germinated in horse dung compared to cattle dung (*H. nummularium*

(scarified seeds), *Tuberaria guttata* (both categories), *Trifolium pratense*, *Trifolium repens*, *Juncus effusus*, *Agrostis stolonifera* and *Poa annua* in greenhouse conditions and *A. stolonifera*, *T. repens* and *T. guttata* (both categories) in outdoor conditions). In the outdoor experiment, significantly more seeds of *T. guttata* (scarified seeds), *T. repens*, *J. effusus*, *Agrostis capillaris*, *P. annua* and *Urtica dioica* germinated in the control treatment compared to cattle and/or horse dung addition treatments. Most species germinated more abundantly in greenhouse conditions, except for *A. stolonifera* where the opposite effect was found while no effect was found for *Alopecurus myosuroides*, *H. nummularium* (scarified seeds) and *P. annua* (Figure 3.2).

Table 3.2 - Results of nested ANOVAs with consecutively germinability and mean time to germination (MTG) as measured variable and dung type, life strategy, environment and family as nominal variables and a nested family: species term.

measured variable	factor	*df*	*F* value	*p*
germinability	dung type	2	155.697	<0.001
	life strategy	1	0.362	0.548
	environment	1	71.21	<0.001
	family	6	239.492	<0.001
	family: species	6	115.569	<0.001
MTG	dung type	2	24.79	<0.001
	life strategy	1	32.201	<0.001
	environment	1	426.897	<0.001
	family	6	88.397	<0.001
	family: species	6	13.61	<0.001

Survival analysis pointed out that seeds sown in the control treatment had a higher probability to germinate than seeds sown in dung covered pots (Figure 3.3). Intact seeds of *H. nummularium* and *A. myosuroides* were less likely to germinate in cattle dung while seeds of all other tested species had a lower germination probability in both horse and cattle dung. Compared to the control treatment in greenhouse conditions, the germination rate was significantly slower for most species when sown in dung or in outdoor conditions (Figure 3.4). One exception was found with *A. stolonifera*. This species germinated significantly faster when sown in outdoor conditions irrespective of the addition of dung. In most cases, germination was hindered most when cattle dung was added to the pots (Figure 3.4).

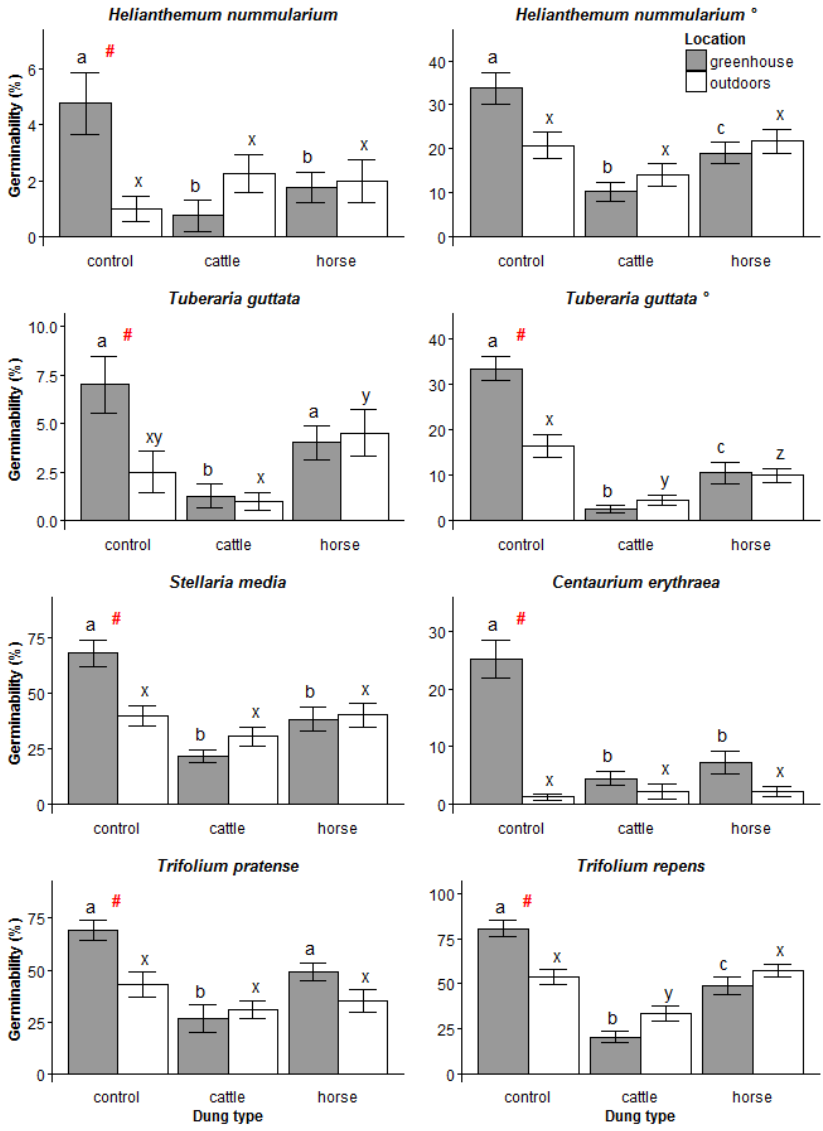

Figure 3.2 - Germinability by dung type and species. Grey bars indicate greenhouse conditions, white bars indicate outdoor conditions. Different letters above bars indicate significant differences between dung types and the control treatment after applying two-way ANOVAs and Tukey posthoc tests on germination data in either greenhouse (a-c) or outdoor (x-z) conditions. Significant differences in germinability between the greenhouse and outdoor environment are marked with # for each particular dung type. Seeds of species indicated with ° were pre-treated by scarification with sandpaper.

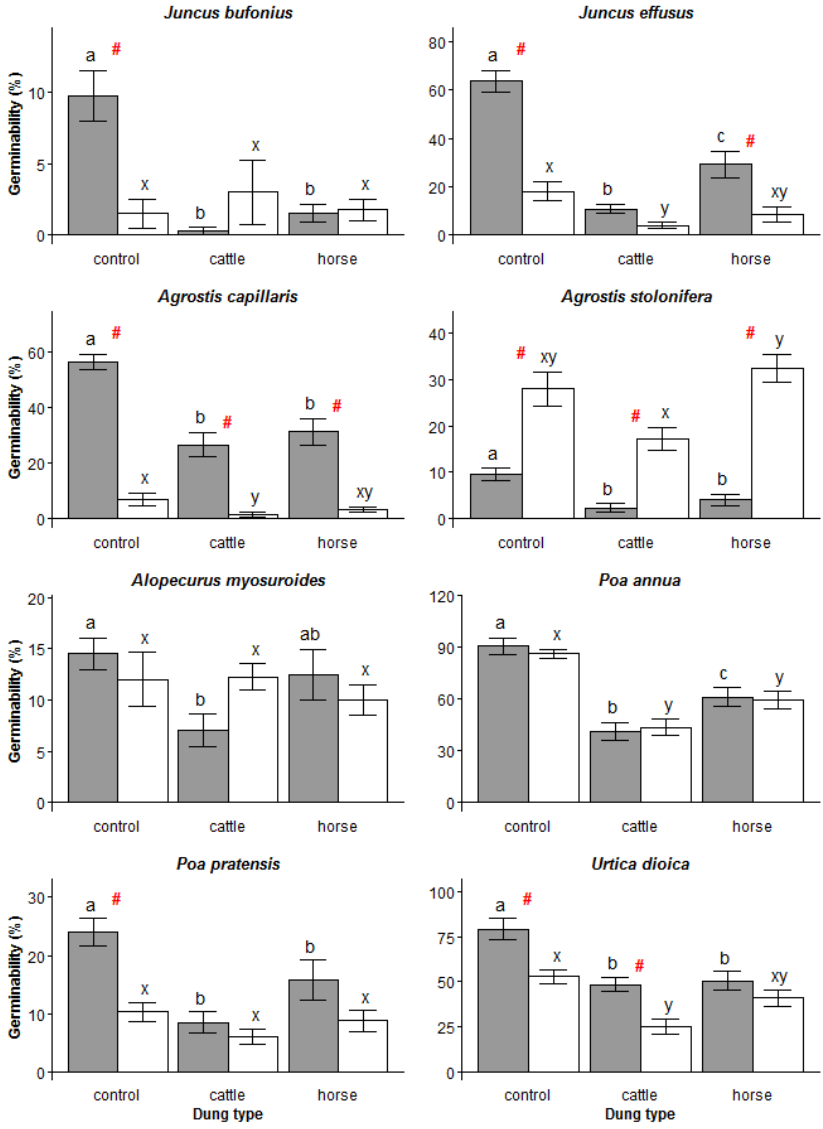

Figure 3.2 continued.

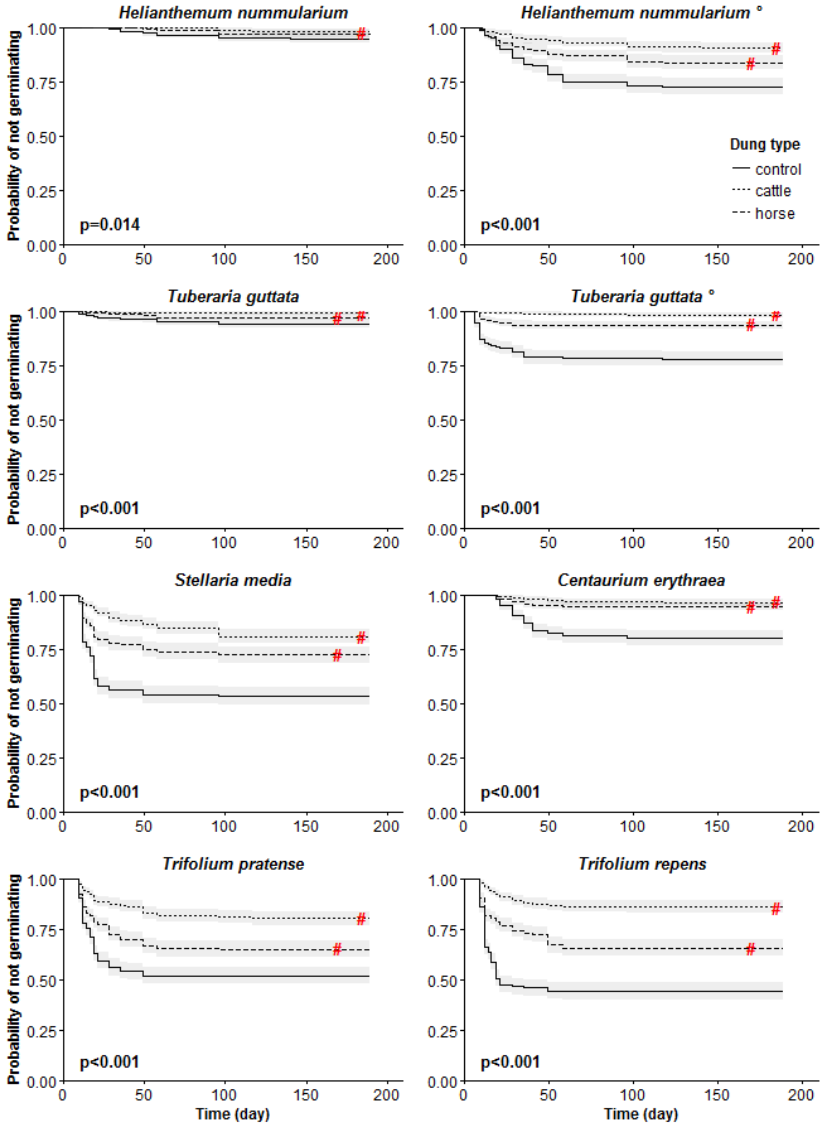

Figure 3.3 - Probability of not germinating (Kaplan-Meier estimates) of germination functions by species sown in greenhouse conditions. Treatment curves which differ significantly from the control treatment after applying AFT-models are indicated with #. Confidence bands are indicated in grey. Seeds of species indicated with ° were pre-treated by scarification with sandpaper.

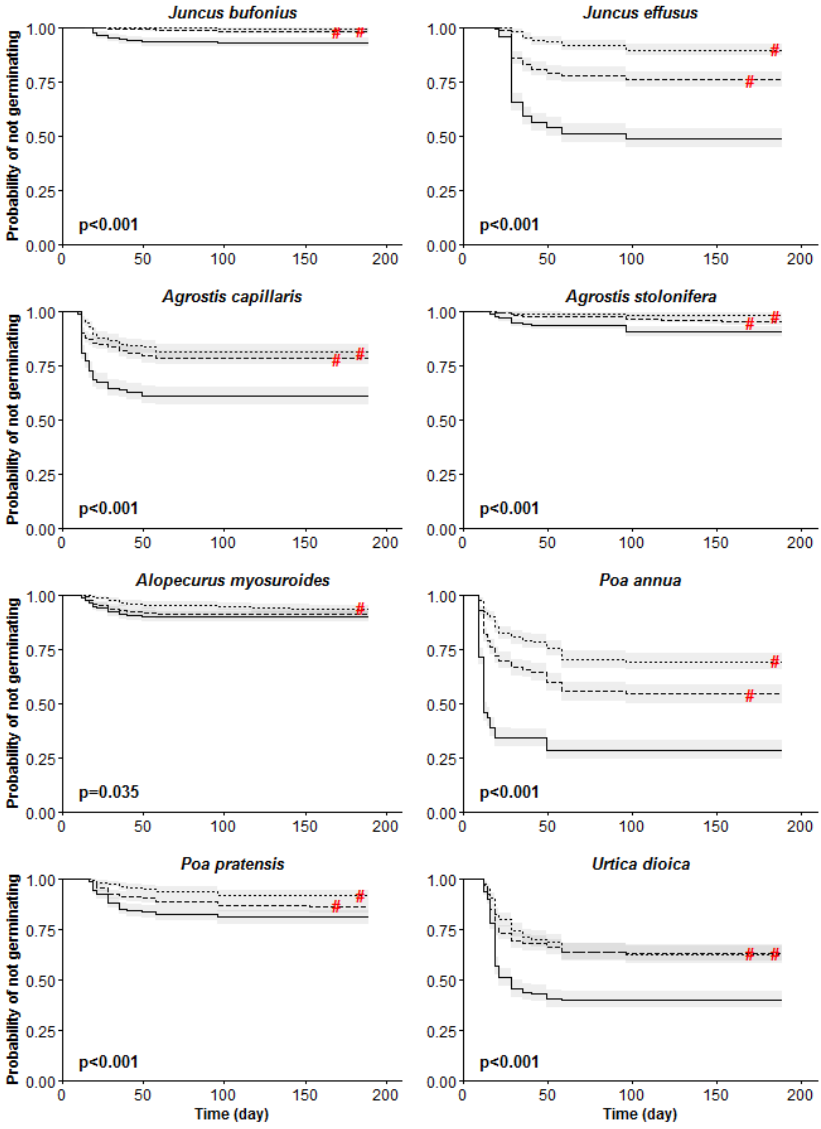

Figure 3.3 continued.

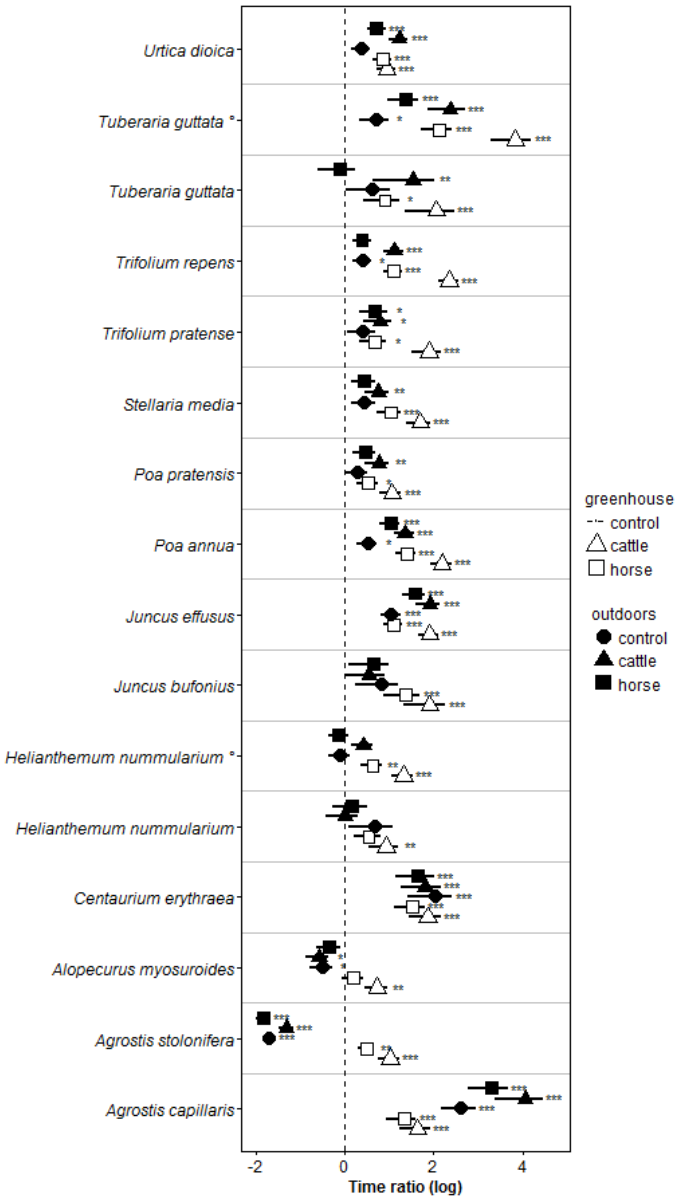

Figure 3.4 - Log-transformed time ratio (TR) and standard errors by species and dung type after applying AFT models. log(TR)>0 indicates decelerated germination and log(TR)<0 indicates accelerated germination compared to the control treatment (log(TR)=0) in greenhouse conditions. Asterisks indicate significance levels with ***: $p<0.001$, **: $0.001<p<0.010$ and *: $0.010<p<0.050$. Seeds of species indicated with ° were pre-treated by scarification with sandpaper.

Discussion

The germination metrics are clearly species-dependent as germinability ranged between 1 and 98% and seeds on average needed between 1.5 and 203 days to germinate in the laboratory test. Except for *Stellaria media*, *Agrostis capillaris* and *Urtica dioica*, more seeds germinated in the laboratory test compared to the control treatments in the greenhouse and outdoor environments although for all species seeds from the same seed lots were used. Similar results were previously found by Ramos-Font et al. (2015) and are likely to be linked to the more favourable combination of light quality, day-night cycle length, substrate and constant temperature and humidity in the lab. Additionally, in the laboratory tests in Petri dishes, it was far more easy to detect germination (appearance of a radicle) than in sand-compost filled pots in the greenhouse or outdoor experiments (appearance of cotyledons). Subtle differences in germination timing were found between life strategies. Annual species generally germinated faster than perennial species which is comparable with the findings of Shipley and Parent (1991) although we did not find different germinability.

For most species the greenhouse conditions were more favourable for germination as more seeds germinated and the time ratio for the outdoor control treatment was significantly higher in most cases. *Agrostis stolonifera* was a clear exception as for this species significantly more seeds germinated at a much faster pace in the outdoor treatment. The environmental conditions differed between both experimental locations and may have triggered different germination responses. In the first weeks of the experiment, the cumulative growing degree-day (GDD) in the greenhouse experiment was higher, which was caused by the higher maximum temperatures, while night temperatures in the unheated greenhouse were comparable with the outdoor conditions. Remarkably, GDD of the soil was much lower in the greenhouse and therefore, the difference between air and soil GDD was smaller in the outdoor experiment. Relative air humidity was highly variable in both locations while in the outdoor location soil humidity had a constant value of nearly 100%, probably due to the high precipitation rate in April 2012. The constantly waterlogged substrate in the outdoor experiment might have triggered germination of *A. stolonifera* which is a native species in disturbed grassland communities with temporarily waterlogged soil and is known to increase its biomass, stolon

length and internodium length in flooded situations (Rozema and Blom, 1977). Alternating temperatures have been linked to several germination metrics (Murdoch et al., 1989; Probert, 2000). Most plant species have an optimal temperature at which maximal germination occurs, with a linear increase towards this optimum. At supra-optimal temperatures, alternating temperatures have little effect while at sub-optimal temperatures germination increases linearly with amplitude (Murdoch et al., 1989). The larger daily fluctuations in temperature in greenhouse conditions might have increased germination in most of the tested species.

Dung clearly has a negative effect on germination. For all tested species, the germination probability is lower in the dung treatments compared to the control treatments. This effect is even more pronounced in the pots covered with cattle dung. In addition to high concentrations of growth-promoting macro- and micro-nutrients, dung may also contain toxic compounds that can have adverse effects on seed survival and germination (Cosyns et al., 2005b; Malo and Suárez, 1995b; Marambe et al., 1993; Ramos-Font et al., 2015; Welch, 1985). Although many seeds survive the intestinal tract and are therefore potentially successfully endozoochorous species, seeds deposited in dung can be destroyed by a wide range of biotic and abiotic factors, including insects, fungi, rodents and desiccation (Estrada and Coates-Estrada, 2002; Vander Wall and Longland, 2004). Besides being a growth substrate for seedlings, dung can also promote the growth of fungi and bacteria which in some cases are detrimental for germination and seedling survival (Clark and Wilson, 2003; Traveset et al., 2007). In our experiments, we observed the rapid development of a hard and dry crust on cattle dung within a few hours after depositing the dung-seed mixture in the pots. According to Holter (2016), both structural and chemical differences between ruminant and non-ruminant dung can be found. Although the water content of both dung types is more or less the same, non-ruminant dung tends to be more fibrous and has larger particles. On the other hand, ruminant dung on average has higher nitrogen content and a lower C: N ratio (Holter, 2016). The more loose structure of horse dung might facilitate the emergence of seedlings. The impact of the higher nitrogen content of fresh ruminant dung is likely to be less important as only a small fraction of the nutrients present in fresh dung is inorganic and readily available for plant uptake (Jørgensen and Jensen, 1997; Sitters et al., 2014). During the experiment, coprophilous fungi appeared on both dung types. Although

fungi are known to increase the decomposition rate of dung (Harper and Webster, 1964), little dung was degraded by the end of the experiment which was probably due to the absence of soil fauna in the plant pots.

Germinability and MTG clearly differed between plant species and families. The tested species in the Urticaceae, Fabaceae and Caryophyllaceae germinated typically fast and abundantly, while few seeds of the Cistaceae and Gentianaceae germinated in any of the experimental treatments. Successful endozoochorous dispersal has been especially linked with species carrying persistent, small, rounded diaspores with a hard seed coat (Albert et al., 2015b). Many species in the Cistaceae and Fabaceae have a high incidence of dormant seeds due to their hard and water impermeable seed coats (D'hondt et al., 2010; Gresta et al., 2007; Thanos et al., 1992). Mechanical scarification of the hard seed coat induces germination (Delgado et al., 2008; Pérez-García and González-Benito, 2006). This observation is confirmed in our experiment with higher germinability of scarified *H. nummularium* seeds compared to intact seeds. Physical dormancy also occurs in *Trifolium* species. D'hondt et al. (2010) found that up to 35% of all viable seeds are water-impermeable in wild populations of *T. repens*. In our experiment, the seeds of both tested *Trifolium* species were not pre-treated but nevertheless had a high germinability. This in line with other experiments using cultivated weed seeds (Milotić and Hoffmann, 2016b) and is probably due to the fact that seed dormancy is a hereditary trait in this species although the environmental conditions during seed maturation also have an effect (Baskin and Baskin, 2001). We should also keep in mind that we used intact, undigested seeds. Germination success decreases in many species after gut passage (e.g., Cosyns et al. (2005b), Grande et al. (2013), Manzano et al. (2005), Milotić and Hoffmann (2016b), Mouissie et al. (2005b)), so we can safely assume that an even lower germination success would have been found when seeds would have been precedingly digested.

We were unable to estimate whether the seeds that remained ungerminated at the end of the experiment were dormant or dead due to the substrates used, given the fact that the substrate did not allow the retrieval of the tiny seeds. However, as Cistaceae species are known to spread germination over several seasons (Russi et al., 1992), we can assume that a reasonable fraction of the seeds remains dormant, even after mild scarification. Endozoochorous dispersal is known to enrich and alter the species composition of soil seed banks by incorporating seeds that remain

dormant following dispersal (Dai, 2000; Nathan and Muller-Landau, 2000). Early emergence and establishment in a dung environment may be an advantage for certain species and even a difference in germination timing of a few days may decrease the growth rate and survival probability of later emerged seedlings (Loiselle, 1990; Traveset, 1998). On the other hand, early germinated seeds might be more susceptible to pathogens, seed predators and the adverse environmental conditions in dung. Therefore, selection is not always in favour of early germination or high germination percentages (Janzen, 1984; Traveset, 1998). Seeds germinating late are able to escape the scramble competition for space and nutrients with other seedlings in the same dung pat. The activity of dung inhabiting fauna whose dung removing and mixing actions may kill or harm vulnerable seedlings decreases with ageing dung (Janzen, 1984). Furthermore, the nutritive content is more readily available for plant uptake in older dung (Jørgensen and Jensen, 1997; Sitters et al., 2014) which might benefit the growth of seedlings that germinated later**.** The large dung pats produced by large herbivores such as cattle and horses, are able to kill or suppress the vegetation underneath the dung pat and, therefore, create gaps with relatively low competition between seedlings and the established vegetation (Brown and Archer, 1989). Therefore, the ideal germination timing after endozoochory is a trade-off between the reduced competition early after deposition and the lowered concentration of phytotoxic compounds and the higher availability of nutrients in a later stage. We should keep in mind that germination is just the first of many steps in plant regeneration. The high nutrient content in dung might alter plant fitness in a later stage and enhance growth and/or reproductive success.

Conclusion

We found adverse effects of both cattle and horse dung on the germinability of all tested grassland species, but the effects of cattle dung were more pronounced. Also, germination metrics clearly differed between the artificial greenhouse and outdoor conditions, with in general lower germinability in outdoor conditions. A large cost seems to be related to endozoochorous dispersal, at least in the first stages of seed survival and germination.

Acknowledgments

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4 Cost or benefit for growth and flowering of seedlings and juvenile grassland plants in a dung environment

Tanja Milotić and Maurice Hoffmann

Plants growing in dung in a greenhouse environment.

Modified from Milotić, T. and Hoffmann, M. (2016) Cost or benefit for growth and flowering of seedlings and juvenile grassland plants in a dung environment. Plant Ecology, **217**(8), 1025-1042, DOI: 10.1007/s11258-016-0629-2

Abstract

Endozoochory is a potential dispersal mode for numerous plant species. Although germination following endozoochory is well-documented, less is known about the costs and benefits associated with this dispersal mode in later life stages of established plants. The chemical and physical nature of dung differs between herbivores and might have specific effects on seedling establishment, growth and flowering. We conducted a growth experiment using 12 temperate grassland species with a known potential for endozoochory. We studied the effects of cattle and horse dung on the juvenile, growth and reproductive phase. Being a ruminant and a hindgut fermenter respectively, cattle and horses are two physiologically contrasting herbivore species, producing structurally quite different dung types. They are additionally interesting model species as both are frequently used in temperate Europe grassland management. Seedling biomass and growth rate, height, ramification, flowering and biomass of grown plants were measured in an attempt to quantify the benefits of endozoochorously dispersed seeds compared to seeds dispersed by other means and thus growing in a virtually dung-free environment. Few species were affected by the presence of dung in the juvenile phase while most species generally benefitted from being deposited in dung in later life stages. Positive responses of *Agrostis capillaris*, *Agrostis stolonifera*, *Alopecurus myosuroides*, *Helianthemum nummularium*, *Poa annua*, *Trifolium repens* and *Trifolium pratense* were found, while dung had a negative effect on *Juncus bufonius*. The initial losses of viable seeds through the digestive system of herbivores might, therefore, be partially compensated by enhanced growth and flowering in some species.

Keywords: dung, endozoochory, grassland species, growth, seedling and juvenile stage

Introduction

Endozoochory, or the dispersal of diaspores through animal guts, is a potential dispersal mode for numerous plant species. Over the recent decades, an increasing number of studies have estimated the endozoochorous dispersal potential of plants in a wide array of ecosystems. In semi-natural landscapes, such as West European temperate grasslands, ungulates are known to be effective seed dispersers (Albert et al., 2015b; Cosyns et al., 2005b; Welch, 1985). Due to the combination of long gut retention times, large gut capacity, high travel velocity and spatial habitat preference patterns, endozoochory by large herbivores could be one of the main mechanisms of long-distance dispersal, enabling directed transport towards suitable habitats (Cousens et al., 2010; D'hondt et al., 2012; Nathan et al., 2008). It is, therefore, an important mechanism in nature management and restoration (Mouissie et al., 2005c; Traba et al., 2003). Although viable seeds have been found abundantly in dung, and hence extensive proof has been provided of the endozoochorous dispersal potential of many grassland species (e.g., Bakker and Olff (2003), Cosyns et al. (2005b), Malo et al. (2000), Malo and Suárez (1995b), Pakeman et al. (2002)), few studies have examined the potential benefits of the nutritive dung environment for further plant development.

In addition to the high concentration of growth promoting macronutrients (N, K and to a lower extent P, Ca and Mg) and essential trace minerals (e.g., Fe, Mn and Cu) (Haynes and Williams, 1993; Lupwayi et al., 2000), dung also contains low concentrations of phytotoxic compounds such as phenols and fatty acids which inhibit germination and initial growth (Berendse and Möller, 2009; Farnsworth, 2008; Luna and Moreno, 2009). Only a small fraction of the nutrients present in fresh dung is inorganic, which implies that the organic compounds must be mineralized before being available for plants (Jørgensen and Jensen, 1997; Sitters et al., 2014). Although the leaching of nutrients from dung to the underlying soil has been described in many ecosystems (e.g., Aarons et al. (2004), Haynes and Williams (1993)), the rates at which nutrients are released are highly variable and depend on factors such as humidity (Dickinson and Craig, 1990; Dickinson et al., 1981), chemical composition of dung (Ouédraogo et al., 2004), vegetation type of deposition sites (Shepherd et al., 2000), but also on the presence of dung fauna such as dung beetles (Lovell and Jarvis, 1996; Yokoyama et al., 1991).

The high moisture content of fresh dung can promote early germination of fast-germinating species (Traveset et al., 2001) while ageing dung gradually becomes drier until a dry crust is formed on the surface which can even resist dung manipulating fauna (Galante et al., 1991; Lumaret et al., 1993) and impedes the imbibition of slower germinating species (Brown and Archer, 1989). Dung deposited in large and dense volumes such as cattle dung, often smothers and kills the underlying vegetation due to the lack of light during the decomposition period (Williams and Haynes, 1995). Furthermore, large depositions of dung are usually avoided by grazing herbivores (Castle and MacDaid, 1972) and hence endozoochorously dispersed seeds could benefit from reduced competition with the existing vegetation, lowered grazing risk and the nutrients that are present in the dung pat. The deposition of seeds in dung is known to alter germination and early seedling growth (Carmona et al., 2013; Traveset et al., 2001), but few studies examine the effects of dung in the later growth stages of grassland plants. The post-dispersal fate of endozoochorically dispersed plant species, such as seedling establishment, survival and adult fitness, remain largely unknown. Especially in long distance dispersal, these processes are vital for the establishment success of new populations at new locations and are probably triggering the development of plant traits that promote one dispersal mechanism above others (D'hondt and Hoffmann, 2011).

Numerous examples of fertilization effects on plants are known, although most of these studies focus on production gains in agricultural systems and few examples are known of possible effects of the presence of dung regarding the establishment success of endozoochorously dispersed seeds in natural ecosystems. Some field studies take the presence of nutrients after endozoochorous dispersal into account (e.g., Bakker and Olff (2003), Cosyns et al. (2006)), but don’t exclude the potential effects of competition with established vegetation beneath dung pats, seeds germinating from the seed bank and inter-seedling competition. Nevertheless, fertilization effects on seedling emergence (Deshaies et al., 2009), aboveground and belowground biomass (Deshaies et al., 2009), seed production (Greipsson and Davy, 1997), seedling establishment (Greipsson and Davy, 1997), phenology (Nomura and Kikuzawa, 2003; Nord et al., 2011; Power et al., 2006) found in other types of studies could also be of importance in the cost-benefit balance of endozoochorous dispersal.

We conducted a growth experiment using 12 temperate grassland species that are commonly found germinating in dung. We studied the effects of cattle and horse dung, two herbivore species commonly introduced in grassland management and with opposing physiology (ruminant versus hind-gut fermenters), on the juvenile, growth and reproductive phase of plants. Seedling biomass, growth rate, height, ramification, flowering and biomass of grown plants were measured in an attempt to quantify the benefits of endozoochorously dispersed seeds compared to seeds dispersed by other means and thus growing in a dung-free environment.

We hypothesized that:

1. the phytotoxic compounds which are present in fresh dung negatively affect plants in their first developmental stages which would be translated in lower seedling biomass and decreased growth rate while
2. once established, seedlings benefit from a gradual increase of inorganic nutrients as the dung starts to decompose which in turn leads to an increase in adult plant height, ramification, biomass and flower number in fertilized plants.
3. We also expected to find interspecific differences in plant growth and flowering, with higher plant height and more flowers in nitrophilous species grown in dung.
4. Finally, due to the structural differences between cattle and horse dung, plant responses might also differ between dung types. The rapidly changing structure of cattle dung from being viscous to a firm and hard to penetrate structure might result in slower seedling establishment compared to seedlings growing in dung types with a loose structure such as horse dung.

Materials and methods

Plant species

12 grassland species belonging to 5 plant families were used in the experiment. In most families both annual and perennial species were selected. Most species are common in temperate European grasslands and are able to disperse endozoochorically (Cosyns et al., 2005a; Cosyns et al., 2005b; Cosyns and Hoffmann, 2005; Couvreur et al., 2005a). The Cistaceae species are less common but were included as earlier research suggested that endozoochory might be an important dispersal mechanism in this family (Mancilla-Leytón et al., 2011; Manzano et al., 2005; Ramos et al., 2006). The distribution of *Helianthemum nummularium* has known a notable increase since the introduction of large herbivores in calcareous dune grasslands in Belgium (Provoost et al., 2015). Besides that, the germination of this species is known to increase after herbivore ingestion (D'hondt and Hoffmann, 2011). *Tuberaria guttata* is an annual Cistaceae species with a Mediterranean distribution (Herrera, 2004) and has been found germinating in dung as well (Malo and Suárez, 1995b; Malo and Suárez, 1996).

Dung addition experiment

Seeds were sown in plant pots with a sand-compost substrate covered with cattle or horse dung (dung treatments) or on a dung free sand-compost substrate (control treatment). Round plastic plant pots (diameter: 15 cm, height: 16 cm) with drainage holes were put in an unheated greenhouse on felt fabric in order to limit drying out. Pots were filled with a 1:1 mixture of sand and compost. Dung was collected from stabled cattle and horses in order to keep contamination with wild seeds minimal. Before sowing, the pots were watered until the soil felt moist. 20 seeds were sown per pot with 20 replicates per treatment with a total of 60 plant pots per species. As species in the Cistaceae family are known for their slow germination rate (Thanos et al., 1992), we doubled the number of replicates to 40 for *H. nummularium* and *T. guttata* in order to assure a statistically appropriate number of plants in the later phases of the experiment. In the treatments including dung, the entire surface of the pots was covered with a 2 cm thick

layer of dung and the seeds were evenly mixed with the dung in order to simulate seed deposition following endozoochory. In the control treatment, seeds were sown directly on the surface in order to simulate unassisted dispersal. Blank pots, with either a top layer of seed-free dung or bare substrate, were installed to check for seed contamination originating from the sand-compost mix, dung or surroundings. All pots were put in a complete randomized design. Pots were watered manually on a daily basis and were subjected to natural daylight cycles. All seeds were sown on April 5th, 2012. After 55 (*Tuberaria guttata*), 60 (*Agrostis capillaris*, *Alopecurus myosuroides*, *Helianthemum nummularium*, *Poa annua*, *Trifolium pratense*, *Trifolium repens* and *Urtica dioica*) or 90 days after the start of the experiment (*Agrostis stolonifera*, *Juncus bufonius*, *Juncus effusus* and *Poa pratensis*), depending on germination and growth rate of each individual species, seedlings were thinned out leaving the tallest seedling in each pot. The removed seedlings were counted and dried at 65°C to constant weight after which dry biomass was measured. From then on, height, ramification and flowering of each individual plant were measured on day 80, 100, 120, 140, 160 and/or 190. *T. guttata* was measured in shorter intervals, at day 60, 70, 90 and 120, as this species has a shorter life cycle (Herrera, 2004). Plant height was measured from soil level to the tallest part of the plant lifting up the hanging shoot parts or tillers. Ramification was quantified by counting branches. Flowering was measured by summarizing the number of flower buds, flowers and fruits. Dry biomass of below and above ground plant parts of flowering species were separately measured. At day 120 (*T. guttata*), 160 (*T. pratense*, *J. bufonius*, *P. annua* and *U. dioica*) or day 190 (all other species), both below and above-ground parts of the plants were harvested and dried to a constant weight at 65 °C.

Data analysis

In each census interval, relative growth rates (RGR) were calculated for each individual plant using plant height:

$$RGR = \frac{\ln(H_t) - \ln(H_{t-\Delta t})}{\Delta t}$$

where H_t is the height at time t, $H_{t-\Delta t}$ is the height in the previous interval and Δt is the length of the time interval (Hunt, 1982). RGR is known to decrease with increasing size and recently it has been shown that non-linear mixed effects modelling leads to a better understanding of plant growth than with the classical approaches (Paine et al., 2012). Therefore, we additionally fitted our height data using nonlinear mixed modelling. Using mixed models, we were able to explore growth curves during the entire experimental period and take into account individual variation by incorporating individual plants as a random factor. The logistic function is most commonly used in plant growth modelling (Paine et al., 2012) and fitted our data best after trying linear, exponential and monomolecular functions. Expected plant height H_t at time t was modelled using non-linear mixed effects models with a 3 parameter logistic base function:

$$H_t = \frac{\varphi_1}{\{1 + exp[-(t - \varphi_2)/\varphi_3]\}}$$

One of the advantages of using this approach is that each parameter in this growth model has a physical interpretation: φ_1 is the asymptotic height; φ_2 is the time at which the plant reaches half of its asymptotic height; and φ_3 is the time elapsed between the plant reaching half and $1/(1+e^{-1})$ ($\pm$ 3/4th) of its asymptotic height (Pinheiro and Bates, 2000). In this model, each replicate was used as a random term, height as the response variable and dung as fixed term. The resulting values for the three parameters (φ_1, φ_2 and φ_3) were compared between the control treatment and both dung addition treatments.

Ramification or number of branches was counted in each census interval. The maximum number of flowers counted on each individual plant was used to quantify flowering success, while the start of the flowering period was used as a time measure. The effect of dung addition on ramification and RGR over time was analyzed with repeated measures ANOVAs with dung

type as fixed effect and individual plants as the error term. If needed, ramification and RGR were respectively log_{10} and *arcsine* square root transformed in order to meet the assumptions for using parametric tests. Kruskal-Wallis tests were used to evaluate the effect of dung on seedling biomass, and flower number, the start of flowering and biomass of flowering plants. Posthoc tests were used to identify significantly differing dung types for each parameter.

Bivariate relationships between plant fitness traits (biomass, ramification and height) and reproductive effort (flower number) were assessed using standardized major axis regressions (SMA). The advantage of using SMA over classical regression techniques is the fact that residual variance in both *x* and *y* dimensions are minimized in SMA line fitting instead of predicting *y* from *x* in the classical approach (Warton et al., 2006). First, the data were tested for common slopes between the dung types. If the slopes did not differ, we tested for common elevations (or y-intercepts) between dung types as the lines fitted to the dung types may both represent a shift along their common slope and/or a shift in elevation (Warton et al., 2012). The relationship between both tested traits was simplified to one regression in case control and dung treatments did not differ in slope and elevation. Models were tested for normality and residual distribution and if needed, data were $\log_{10}$ transformed.

All analyses were performed using R version 3.2.3. (R Core Team, 2015b). The packages 'pgirmess' version 1.6.2. (Giraudoux, 2015), 'nlme' version 3.1-122 (Pinheiro et al., 2015) and 'smatr' version 3.4-3 (Warton et al., 2012) were used for respectively Kruskal-Wallis and non-parametric posthoc tests, non-linear mixed effects modelling and standardized major axis regressions.

Results

Juvenile phase

No seedlings emerged in the blank pots. Juvenile biomass of most tested species was not affected by the addition of cattle or horse dung (Table 4.1). The biomass of seedlings of *Trifolium pratense* grown in horse dung was on average 1.5 times greater compared to the plants grown in cattle dung or on a bare surface while seedling biomass of *Juncus effusus* was decreased sevenfold when grown in cattle dung compared to the control treatment (Table 4.1). *Juncus bufonius* emerged in only 2 pots in the cattle dung treatment, therefore, results of the cattle dung treatment were discarded from further analyses of this species.

Growth phase

Dung treatments differed from controls for a maximum of 2 out of 3 regression parameters, which suggest that the addition of dung only had an effect on specific parts of the growth curve of most species (Appendix, Table A 4.1). The asymptotic height equalling the final height reached by plants was significantly higher for *Helianthemum nummularium*, *Trifolium pratense*, *Agrostis capillaris* and *Agrostis stolonifera* when grown in cattle dung and for *Trifolium repens* when grown in horse dung (Figure 4.1). More time was needed in the first part of the growth curves (X_{mid}) of *H. nummularium* (cattle dung), *J. bufonius* (horse dung) and *J. effusus* (cattle and horse dung), while *Urtica dioica* grew faster when sown in horse dung. The time needed to fulfil the second part of the growth curve, indicated by the scale parameter, was significantly higher for *J. bufonius* (horse dung), while significantly less time was needed for *A. capillaris* (cattle and horse dung) and *U. dioica* (cattle and horse dung) (Appendix, Table A 4.1).

Table 4.1 - Average individual juvenile biomass (g, whole plants) ± standard error and number of pots in which germination occurred between brackets (out of 40 pots for both Cistaceae species and out of 20 pots for all other species). Different letters on the same row indicate significant differences between dung types within 1 species after applying Kruskal-Wallis and posthoc tests.

plant family and species name	life strategy	*p*	control	cattle	horse
Cistaceae					
Helianthemum nummularium (L.) Mill.	perennial	ns	0.014±0.016 (11)	0.010±0.015 (28)	0.012±0.017 (19)
Tuberaria guttata (L.) Fourr.	annual	ns	0.007±0.009 (23)	<0.001±0.001 (6)	0.004±0.008 (13)
Fabaceae					
Trifolium pratense L.	perennial	*p*=0.003	0.087±0.028 (18) a	0.072±0.047 (9) a	0.131±0.056 (18) b
Trifolium repens L.	perennial	ns	0.074±0.022 (19)	0.052±0.047 (12)	0.087±0.052 (9)
Juncaceae					
Juncus bufonius L.	annual	ns	0.130±0.244 (10)	<0.001±0.001 (2)	<0.001±0.001 (3)
Juncus effusus L.	perennial	*p*=0.001	0.015±0.011 (20) a	0.002±0.005 (12) b	0.008±0.009 (15) ab
Poaceae					
Agrostis capillaris L.	perennial	ns	0.072±0.038 (20)	0.065±0.055 (12)	0.068±0.039 (15)
Agrostis stolonifera L.	perennial	ns	0.252±0.286 (14)	0.249±0.256 (2)	0.938±1.316 (5)
Alopecurus myosuroides Huds.	annual	ns	0.080±0.091 (16)	0.070±0.063 (6)	0.191±0.184 (10)
Poa annua L.	annual	ns	0.090±0.022 (18)	0.104±0.060 (17)	0.111±0.055 (19)
Poa pratensis L.	perennial	ns	0.305±0.216 (18)	0.147±0.184 (12)	0.263±0.264 (13)
Urticaceae					
Urtica dioica L.	perennial	ns	0.065±0.022 (19)	0.089±0.083 (20)	0.087±0.046 (17)

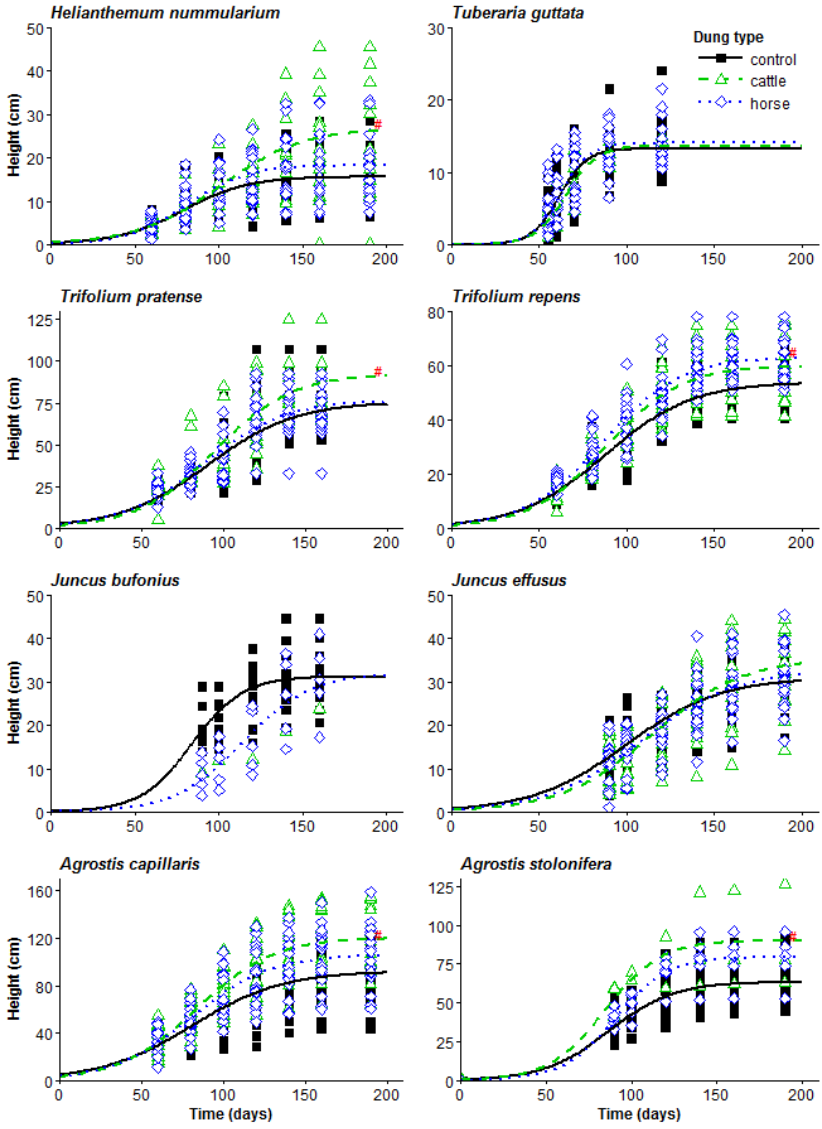

Figure 4.1 - Measured height during the growth and flowering phase by species. Curves were obtained after implementing non-linear mixed effects modelling with a 3 parameter logistic base curve. Treatment curves that differ significantly from the control treatment for the asymptotic height parameter (φ_1) are indicated with #. See Appendix**,** Table A 4.1 for parameter values.

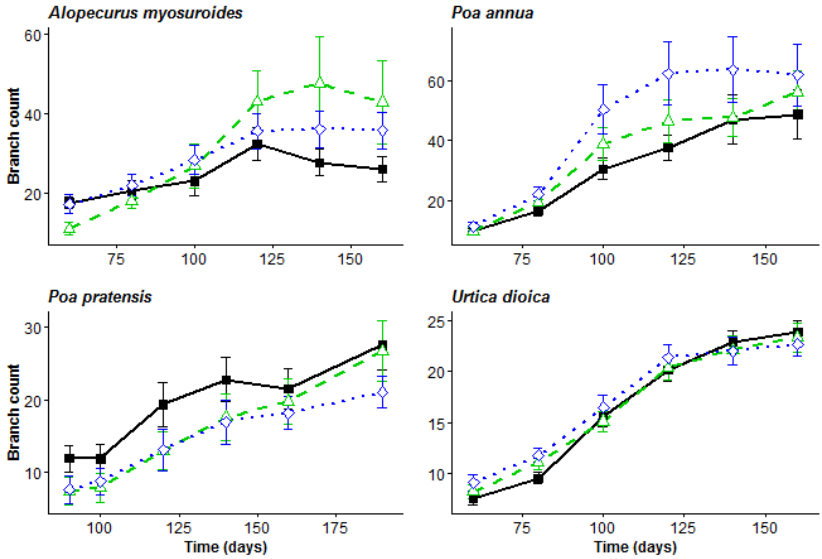

Figure 4.1 continued.

Significant differences in relative growth rate (RGR) between dung treatments were found for *H. nummularium* and *J. bufonius* (Appendix, Table A 4.2). RGR was higher in cattle dung compared to the control treatment (z=3.638; p<0.001) and compared to horse dung (z=-2.466; p=0.036) in *H. nummularium* and higher in horse dung compared to the control treatment in *J. bufonius* (z=6.587; p<0.001) (Appendix, Figure A 4.1). In all species, RGR decreased significantly over time. Relative growth rates differed significantly between species when comparing the control RGRs over the entire growth period (χ^2= 130.53, p<0.001), and allowed to differentiate between fast (*T. guttata* and *U. dioica*), moderately fast (*J. effusus*) and slow growing species (*Poa pratensis*).

Ramification was affected by dung treatments in *A. capillaris*, *A. stolonifera* and *J. bufonius* (Appendix, Table A 4.2). In *A. capillaris,* more branches were counted on the individuals sown in cattle dung compared to the ones in horse dung (z=-2.691; p=0.019). The addition of horse dung resulted in a lower branch count compared to the control treatment in *A. stolonifera* (z=-2.441; p=0.038) and *J. bufonius* (z=-2.419; p=0.016) (Figure 4.2).

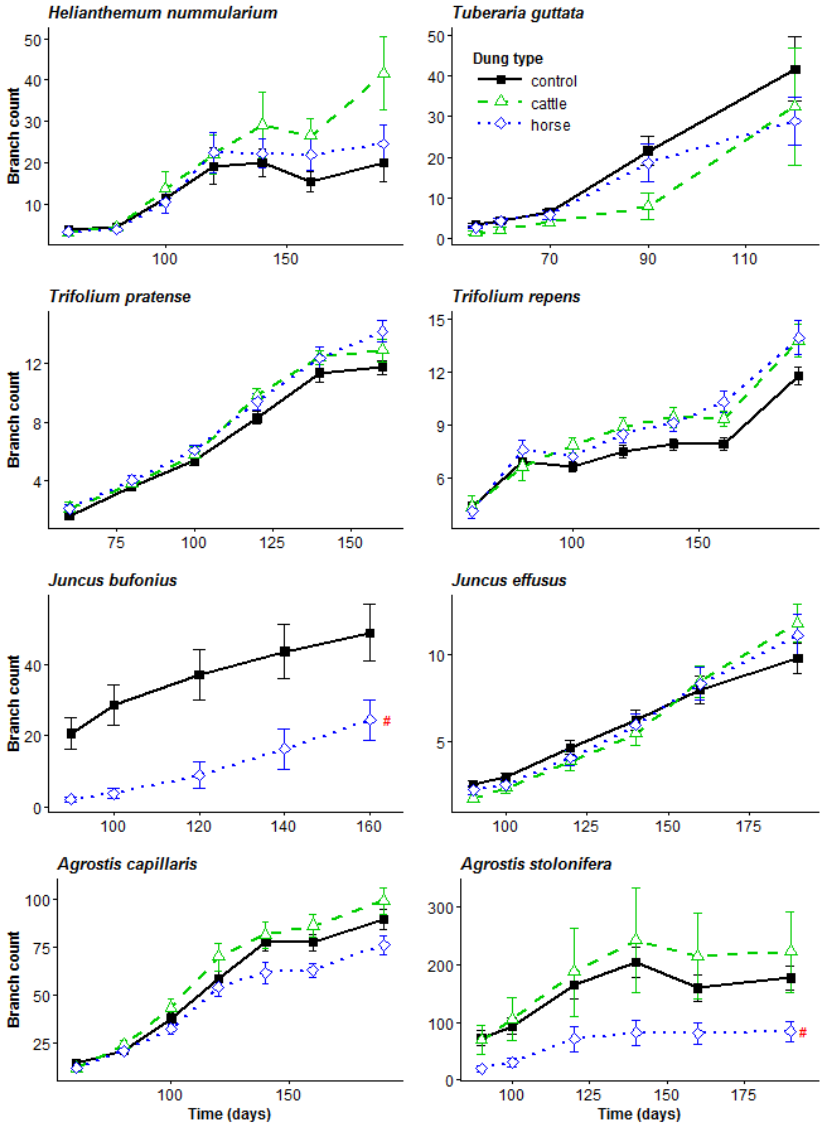

Figure 4.2 - Average branch count during the growth and flowering phase by species and dung type. Dung types significantly differing from the control treatment after applying repeated measures ANOVAs are marked with hashtags (#). Vertical bars represent standard errors. ANOVA results are shown in Appendix, Table A 4.2.

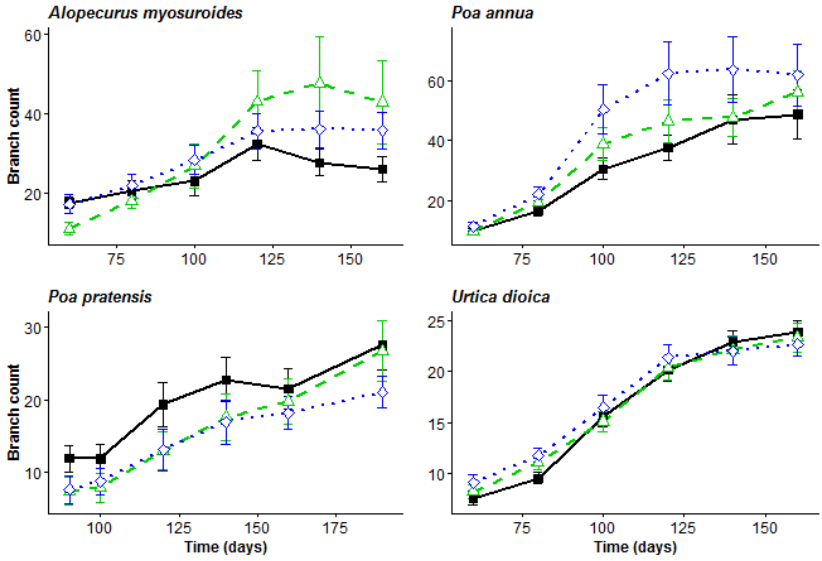

Figure 4.2 continued.

Reproductive phase

During the experimental period, flowers developed on *T. guttata*, *T. pratense*, *T. repens*, *J. bufonius*, *A. capillaris*, *A. stolonifera*, *A. myosuroides*, *P. annua* and *U. dioica* (Table 4.2). As only 1 individual of *A. stolonifera* developed flowers, this species was discarded from further analyses of reproductive traits. When sown in horse dung, *T. pratense* developed significantly more flowers while the plants started flowering at the same time, irrespective of the applied dung treatments. On the other hand, *J. bufonius* developed four times fewer flowers and started flowering later when sown in horse dung compared to the control treatment. The flowering period started later for *A. myosuroides* sown in cattle dung, but at the end of the experiment, reproductive success was comparable between treatments. Dung addition had a positive effect on the individual biomass of *T. repens* (in horse dung) and *A. myosuroides* (cattle dung) while the opposite was found for *J. bufonius*. Nevertheless, neither biomass nor the reproductive success of most of the tested species was affected by the addition of dung (Table 4.2).

Table 4.2 - Mean biomass, maximum flower number and starting date of flowering with standard errors by species and treatment. The number of measured plants is given between brackets. In case treatments differ significantly (p<0.050), p-values are given. In other cases 'ns' is plotted. Different letters on the same row indicate significant differences between dung types after applying Kruskal-Wallis and posthoc tests.

measured variable	species	*p*	control	cattle	horse
biomass (g)	*Alopecurus myosuroides*	*p*=0.037	9.16±1.76 (16) a	21.50±4.67 (10) b	12.81±2.48 (14) ab
	Juncus bufonius	*p*=0.028	3.74±0.66 (12) a		1.46±0.78 (6) b
	Poa annua	ns	11.69±2.72 (15)	12.92±2.59 (17)	16.21±3.20 (16)
	Trifolium pratense	ns	23.03±1.94 (19)	27.83±2.22 (12)	24.87±1.71 (19)
	Trifolium repens	*p*=0.043	18.88±1.15 (20) a	18.66±0.69 (14) ab	22.82±1.31 (19) b
	Tuberaria guttata	ns	0.21±0.21 (18)	0.07±0.06 (4)	0.15±0.12 (15)
	Urtica dioica	ns	14.32±1.83 (19)	13.94±1.70 (20)	12.95±1.73 (20)
maximum flower number	*Alopecurus myosuroides*	ns	25±2 (16)	17±4 (10)	25±5 (14)
	Juncus bufonius	*p*=0.010	394±57 (12) a		91±57 (5) b
	Poa annua	ns	5±2 (15)	11±5 (16)	8±3 (16)
	Trifolium pratense	*p*=0.023	14±1 (19) a	23±3 (12) ab	26±6 (19) b
	Trifolium repens	ns	1±1 (20)	0±0 (14)	3±1 (19)
	Tuberaria guttata	ns	116±95 (18)	51±33 (4)	104±67 (15)
	Urtica dioica	ns	41±7 (19)	64±10 (20)	49±14 (20)
start of flowering (days after sowing)	*Alopecurus myosuroides*	*p*=0.014	87±14 (16) a	106±11 (10) b	96±22 (14) ab
	Juncus bufonius	*p*=0.004	99±13 (12) a		143±8 (3) b
	Poa annua	ns	131±18 (10)	134±16 (13)	134±15 (10)
	Trifolium pratense	ns	110±16 (19)	106±11 (12)	105±11 (19)
	Trifolium repens	*p*=0.033	99±9 (5) a		88±7 (7) b
	Tuberaria guttata	ns	6±6 (18)	69±15 (4)	63±3 (15)
	Urtica dioica	ns	110±23 (17)	99±22 (18)	99±25 (16)

Plant biomass and height had a clear linear relation (R^2=0.749, p<0.001), but this relation was identical for all dung treatments (Figure 4.3). A similar relation was found between above and below ground biomass (R^2=0.183, p<0.001), while different slopes were found between cattle and horse dung (p=0.012) in the SMA analysis using ramification and biomass (Figure 4.3).

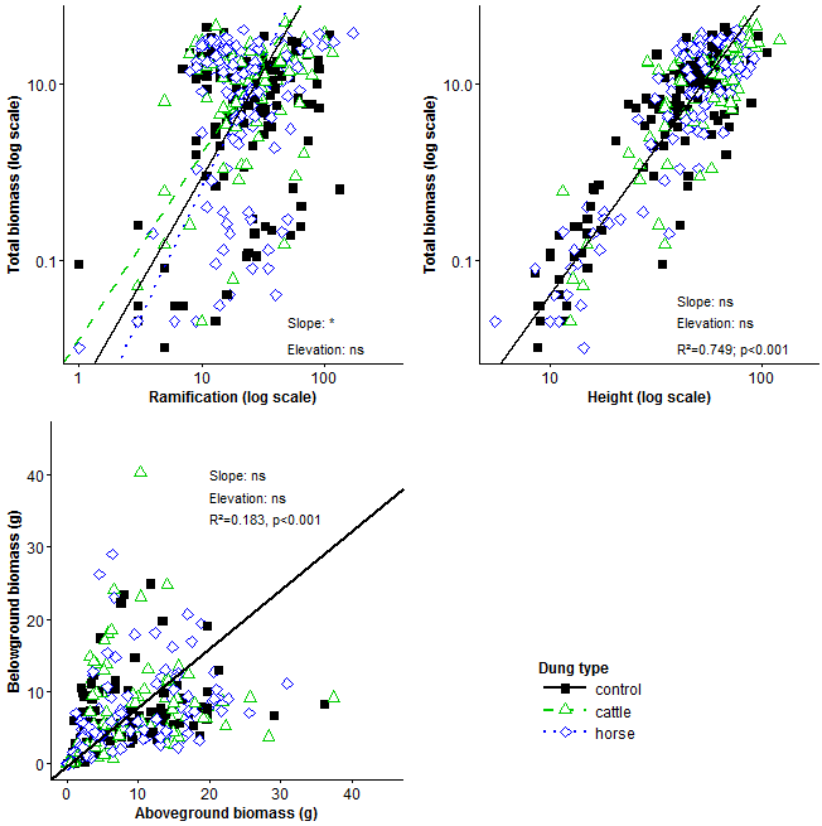

Figure 4.3 - Standardized major axis regressions (SMA) between ramification and biomass (left), height and biomass (middle) and aboveground and belowground biomass (right) across different dung treatments (cattle, horse or no dung). 'Slope' indicates differences in SMA slopes, 'Elevation' indicates differences in SMA elevations (i.e. *y*-axis intercept), significant differences (p<0.050) in slopes and elevations are marked with asterisks, non-significant results are marked with '*ns*'. In case a significant linear relationship between both measured variables is found, but significant effects of dung types are absent, R^2 and p-values for the SMA regression on the whole dataset are plotted.

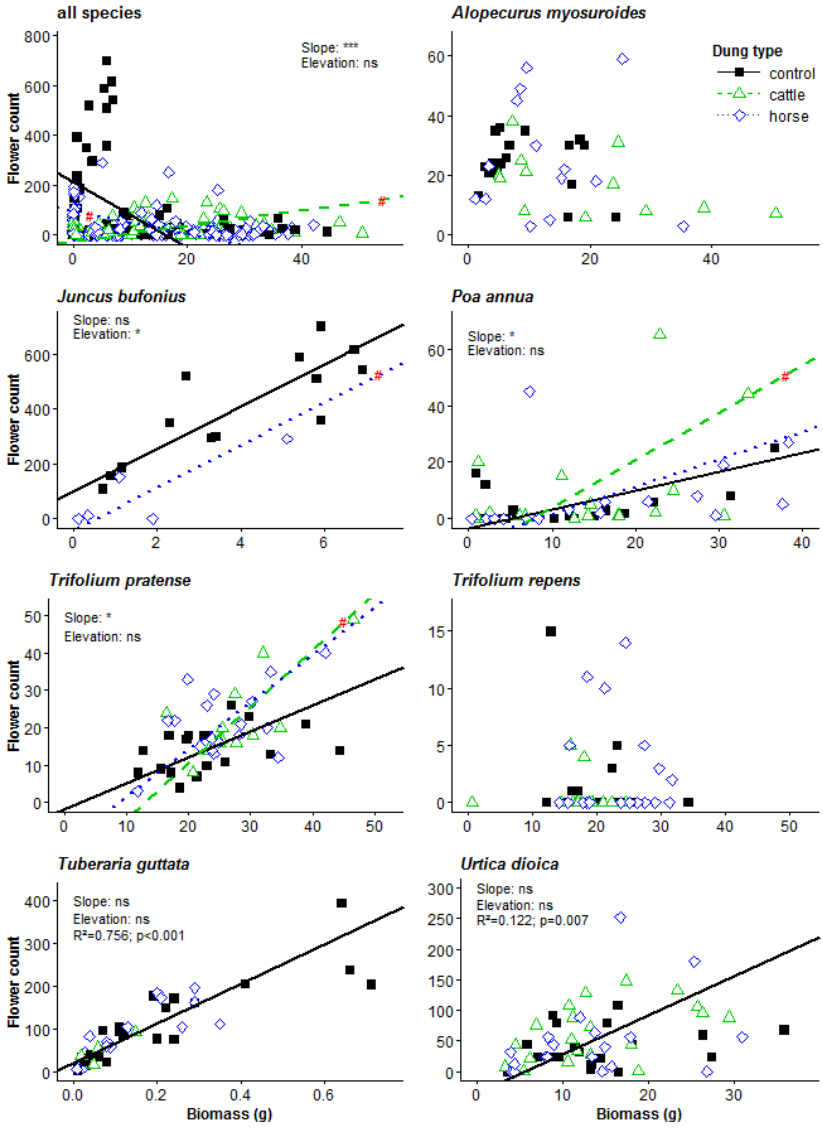

Figure 4.4 - Bivariate plots of standardized major axis regressions (SMA) between biomass and flower number across dung treatments (cattle, horse or no dung) by plant species. 'Slope' indicates differences in SMA slopes, 'Elevation' indicates differences in SMA elevations (i.e. y-axis intercept), significant differences (p<0.050) in slopes and elevations are marked with asterisks and non-significant results are marked with 'ns'. Dung types significantly differing from the control treatment are marked with hashtags (#). In case a significant linear relationship between both measured variables is found, but significant effects of dung types are absent, R^2 and p-values for the SMA regression on the whole dataset are plotted.

A clear linear relation was found between biomass and flower number with different slopes between dung and control treatments (p<0.001 for both cattle and horse dung, Figure 4.3), although this was not the case for all tested species. No linear relation between biomass and flower number was found for *A. myosuroides* and *T. repens* while a positive linear relation was found for all treatments in *T. guttata* and *U. dioica*. Different elevations were found for *J. bufonius* between the control and horse dung treatment resulting in more flowers for a given biomass in the control treatment. Cattle dung and control treatments differed significantly in slope in *Poa annua* (p=0.008) and *T. pratense* (p=0.019) and led to more flowers once the plants reached a certain biomass (Figure 4.4).

Discussion

Few of the tested species were affected by the presence of dung in the juvenile phase while dung affected most of the species in the growth and flowering phase. However, species differed in the nature of this response and the effect of both dung types differed within plant species. *Agrostis capillaris*, *Agrostis stolonifera*, *Alopecurus myosuroides*, *Helianthemum nummularium* and *Trifolium pratense* were positively affected by the addition of cattle dung in one or more growth parameters. Horse dung enhanced the growth of *Trifolium repens* while the opposite was found for *Agrostis stolonifera* and *Juncus bufonius*. Flowering started earlier in *T. repens* when horse dung was added, later in *J. bufonius* (horse dung) and *A. myosuroides* (cattle). More flowers developed on *Poa annua* when cattle dung was applied, but this effect was only apparent for plants with a higher biomass. The growth and flowering of *Juncus effusus*, *Poa pratensis*, *Tuberaria guttata* and *Urtica dioica* were not affected by dung addition.

Early growth and seedling biomass clearly differed among the tested species. Although it was not part of this study, the germination behaviour of our tested species is known to vary (Milotić and Hoffmann, 2016b). While most species exhibited a clear peak in germination timing (e.g., *U. dioica* and the *Trifolium* and *Poa* species) the germination of *H. nummularium*, *T. guttata* and *J. bufonius* was more spread in time and caused the high

variability in seedling biomass in these species. In addition, the shape of the growth curves clearly differs between species, with an earlier response of *J. bufonius* and *T. guttata* and might be related to morphologic traits and life history. Annual species generally have higher seedling growth rates compared to their perennial relatives and differently allocate biomass (Garnier, 1992). Furthermore, small-seeded species have lower resource storage in their seeds and generally have a faster growth rate in their early developmental phases (Gross, 1984; Jurado and Westoby, 1992).

The growth response of plants to nutrient addition is known to vary between species and depends highly on the concentration in which nutrients are presented to plants (Fichtner and Schulze, 1992). Ellenberg (1974) quantified species specific preference for nitrogen by assigning an N-number, a relative measure for nitrophily based on distribution patterns, to individual species. Both relative growth rate and biomass increase with nitrogen concentration available for plant uptake and this effect is more pronounced in plants with a high degree of nitrophily (Fichtner and Schulze, 1992). Most of the species used in our experiment are moderately nitrophilous with an N-number of 4 to 6 on a 1-9 scale, but exceptions are found in *T. guttata* and *H. nummularium* which are most commonly found growing on nitrogen deficient soils (N-numbers of respectively 1 and 2), and in *Poa annua* and *Urtica dioica* which prefer nutrient rich soils (N-numbers of respectively 8 and 9) (Ellenberg, 1974). The poor response of both nitrophilous species might indicate that the decomposition of dung in greenhouse conditions is rather slow and the nutrient levels in the soil are lower than in the natural habitats of nitrophilous species. At the end of the experiment after 190 days, a considerable amount of dung was still visible in the pots. In a field experiment, Jørgensen and Jensen (1997) measured a 10% uptake of dung-N after 16 weeks, while 27% remained on the soil surface in residual dung and 57% was incorporated into the soil through faunal activity. Although the decomposition rate of dung is positively related with irrigation (Dickinson and Craig, 1990; Dickinson et al., 1981) and microbial action in dung pats accounts for a large part of the mineralization of the readily decomposable organic N (Lovell and Jarvis, 1996; Yokoyama et al., 1991), the absence of dung-inhabiting fauna, such as earthworms, dung beetles or isopods, in our experiment might have resulted in slower dung decomposition. Besides nitrogen, large quantities of P, K, Ca, Na, Mg and S leach to the soil from dung pats (Aarons et al., 2004) and the concentration

of macro and micro nutrients is known to vary between herbivore species. On average, slightly higher concentrations of nitrogen, potassium and phosphorus are found in horse dung compared to cattle dung, but within herbivore species, dung nutrient content also varies with diet (Moreno-Caselles et al., 2002). Furthermore, the structural differences between cattle and horse dung could affect the establishment of plants. Whereas a surface crust rapidly appeared on the initial solid manure of cattle, the rather loose structure of horse dung was conserved during the entire experiment.

The effects of dung addition on plant establishment, growth and flowering are complex as not only the structural composition and the nutritive quality of dung varies with herbivore species and diet, but different concentrations and proportions of macro and micro nutrients could have a species-specific effect on plant development as well (Dai, 2000; Jørgensen and Jensen, 1997). Although nutrients leach in high concentrations from dung pats from the early phases of decomposition onwards, particularly if sufficient moisture is present (Dickinson and Craig, 1990), dung had few effects on juvenile biomass in our experiment. Seeds contain a reserve of mineral and organic nutrients to nourish the embryo in its initial stages of establishment, but once this storage is depleted seedlings have to use the available nutrients in the soil (Fenner and Thompson, 2005). We should also keep in mind that we used commercially available potting compost which is supplied with nutrients for the first 6-8 weeks (1.25 kg/m³ with N-P-K concentrations of 14-16-18). Although the actual nutrient levels of the substrate were only half as we made a custom mix of 1:1 sand and compost, we can assume that nutrients were not limiting in the control treatment during the first phase of the experiment. Furthermore, seedling growth was not constrained by the phytotoxic compounds that are often present in fresh dung (Hoekstra et al., 2002). Carmona et al. (2013) found species-specific effects in the growth response of seedlings to dung leachates. Species known to increase their distribution under grazing developed longer roots when dung leachates were provided while the opposite effect was found in species that are less resistant to herbivores. The mainly positive or indifferent response to dung addition of our tested species in the growth and flowering phase could be related to the fact that most species are commonly found in dung and can be considered as increaser species under grazing. *H. nummularium* is such an increaser species since the distribution of this normally rare species increased remarkably after the introduction of large herbivores in

calcareous dune grasslands (Provoost et al., 2015). Although a low N-number has been assigned to this species (Ellenberg, 1974), the cover of *H. nummularium* is known to increase at the edge of cattle dung patches (Dai, 2000) which is also in line with the increased height and growth rate found in our experiment. In contrast, the addition of dung did not affect the growth or reproductive phase of *J. effusus*, *P. pratensis*, *T. guttata* and *U. dioica* although these species have been found germinating in dung, and therefore have a documented link with endozoochorous dispersal (Cosyns et al., 2005a; Cosyns et al., 2005b; Cosyns and Hoffmann, 2005; Couvreur et al., 2005a; Malo and Suárez, 1995b; Malo and Suárez, 1996). On the other hand, since the presence of dung did not hamper the development of these unresponsive species and the germination of these species is hardly affected by simulated herbivore gut passage (Milotić and Hoffmann, 2016b), we can deduce that some species do not seem to experience costs from endozoochorous dispersal.

In terms of endozoochorous dispersal success, most of our tested species seem to benefit from being deposited in dung during the growth and flowering phase. However, we should keep in mind that due to practical reasons we used intact, untreated seeds while in case of endozoochorous dispersal seeds undergo a sequence of mechanical, physical and thermal processes (see Milotić and Hoffmann (2016b)). Previous studies refer to the contrasting effects of gut passage on seed germination success which increases in some species (e.g., D'hondt and Hoffmann (2011), Grande et al. (2013), Mancilla-Leyton et al. (2011), Manzano et al. (2005), Ramos et al. (2006), Ramos-Font et al. (2015)) while the opposite was recorded for other species (e.g., Cosyns et al. (2005b), D'hondt and Hoffmann (2011), Grande et al. (2013), Manzano et al. (2005)). Feeding experiments previously resulted in a decreased survival of respectively 50, 70, 20, 75 and 70% of *A. capillaris*, *A. stolonifera*, *J. bufonius*, *P. annua* and *P. pratensis* seeds, while survival rate increased respectively with 10 and 5% in *H. nummularium* and *T. pratense* (D'hondt and Hoffmann, 2011). Lower germinability was found after a simulated digestion experiment using cattle and horses as model organisms for *T. pratense* and *T. repens*, while digestive processes did not seem to hamper the germinability of *H. nummularium*, *P. annua*, *P. pratensis*, *T. guttata* and *U. dioica* (Milotić and Hoffmann, 2016b). Furthermore, Cosyns et al. (2005b) found a decreased germination success

of undigested seeds when sown in dung compared to bare soil (which was the case for e.g., *A. capillaris*, *H. nummularium* and *P. pratensis*).

Endozoochorous dispersal success is closely related to plant traits such as diaspore size and shape, permeability or robustness of the seed coat and seed longevity (Albert et al., 2015a; Albert et al., 2015b; Bruun and Poschlod, 2006; Pakeman et al., 2002). Besides slowing down germination (Ramos-Font et al., 2015), the presence of dung might also reduce establishment success by facilitating fungal and bacterial growth while in contrast it might protect against specific predators and parasites (Fragoso et al., 2003; Meyer and Witmer, 1998; Traveset et al., 2007). The reduced germinability after digestion by ungulates (Cosyns et al., 2005b; Gardener et al., 1993b; Peco et al., 2006a) combined with a decreased germination success in dung (Cosyns et al., 2005b) suggests that the first steps in the endozoochorous dispersal process represent a major bottle-neck for overall dispersal success of grassland plants. Although we used intact seeds from the same seed lot in both the control and dung treatments, we can expect that the different germination timing of digested seeds would affect seedling growth and plant fitness (Verdú and Traveset, 2005). Therefore, the results of our experiments should rather be interpreted as the outcome of one particular step in the endozoochorous dispersal process instead of being a proxy for the complete mechanism of endozoochory.

On the other hand, in a natural environment, the benefits for endozoochorously dispersed seeds might be more pronounced as in our experiment competition with established vegetation was ruled out. The large faecal depositions of horses and cattle may smother and kill the existing vegetation and thereby create gaps for the establishment of opportunistic species (Castle and MacDaid, 1972; Janzen, 1984; Williams and Haynes, 1995). In temperate grasslands which typically consist of dense turf, the establishment of seedlings requires, at least, some degree of disturbance in order to reduce competition with older plants (Bullock et al., 1994; Fenner and Thompson, 2005; Watt and Gibson, 1988). Furthermore, seeds buried too deep in the dung pat might not germinate due to light deprivation as long as the dung pile remains intact (Bliss and Smith, 1985), and get incorporated in the soil seed bank (Jaroszewicz, 2013; Malo et al., 1995; Pakeman et al., 1999) while escaping the scramble competition for nutrients and space with other seedlings in the same dung pat. In our experiment, dung layers were only 2 cm thick which at least allowed the

germination of a fraction of the sown seeds of the test species, but as the seeds in the control pots were sown directly on the substrate as it would be in case of undirected dispersal, this might have had an effect on germination timing and early seedling growth. Seeds are more likely to germinate when located in the top layers of the soil where light is able to penetrate and temperature is variable (Bliss and Smith, 1985; Galinato and Van der Valk, 1986; Limón and Peco, 2016; Traba et al., 2004; Woolley and Stoller, 1978) and become dormant when sown too deep (Benvenuti et al., 2001). Furthermore, this response is size-dependent as heavier seeds are able to germinate in deeper layers (up to 8 cm deep), while light seeds should not be sown deeper than 1 cm (Benvenuti et al., 2001; Galinato and Van der Valk, 1986; Grundy et al., 2003; Limón and Peco, 2016). In this respect, the germination of small-seeded species such as *T. guttata*, which is triggered by light at a maximum depth of 2mm (Benvenuti et al., 2016), could be hampered by our experimental set-up.

Extensive grazing generally leads to an increase in structural diversity and species richness in a direct way through the selective consumption of palatable plant species, but indirectly the similarity between grazed communities and the variation within communities is enhanced through the deposition of dung patches and the specific growth responses of endozoochorously dispersed species compared to other seed dispersal mechanisms (Malo and Suárez, 1995a; Olff and Ritchie, 1998; Peco et al., 2006b).

We can conclude that grassland species with a known link to endozoochorous dispersal generally benefit from the deposition in dung, although this effect is only apparent in later life stages. Whereas no apparent differences were found between the tested dung types, plant species did differ in their response to dung addition. The initial losses of viable seeds through the digestive system of herbivores might, therefore, be at least compensated by enhanced growth and flowering in some species.

Acknowledgments

We would like to express our gratitude to the Flanders Marine Institute (VLIZ) for the permission to use their greenhouse facilities and to Frank Broucke, Michiel T'Jampens and André Cattrijsse for technical assistance and the daily watering of the greenhouse experiments. We would also like to thank Bjorn Jansseune for providing seed- and drug-free dung. Finally, we would like to pay tribute to the two anonymous reviewers for their helpful comments and suggestions to improve previous versions of this paper.

Appendix

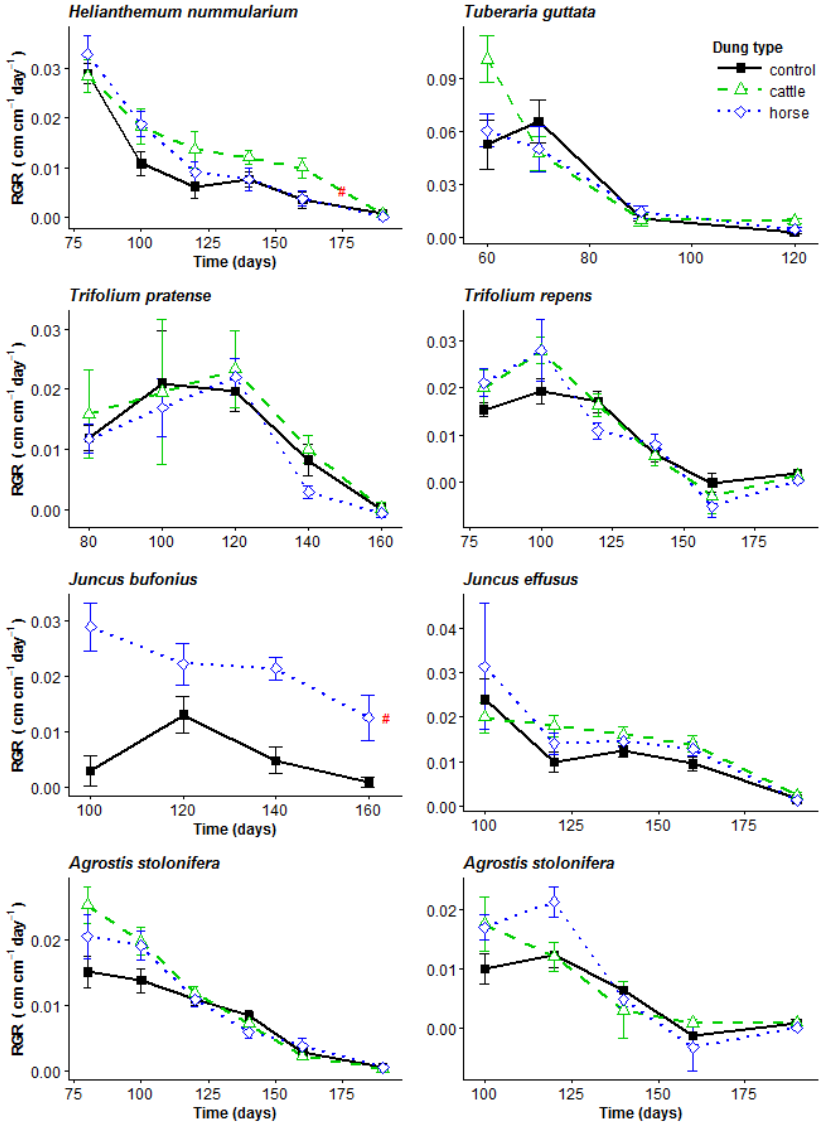

Figure A 4.1 - Average relative growth rate (RGR) during the growth and flowering phase by species and dung type. Dung types significantly differing from the control treatment after applying repeated measure ANOVAs are marked with hashtags (#). Vertical bars represent standard errors. ANOVA results are shown in Table A 4.2.

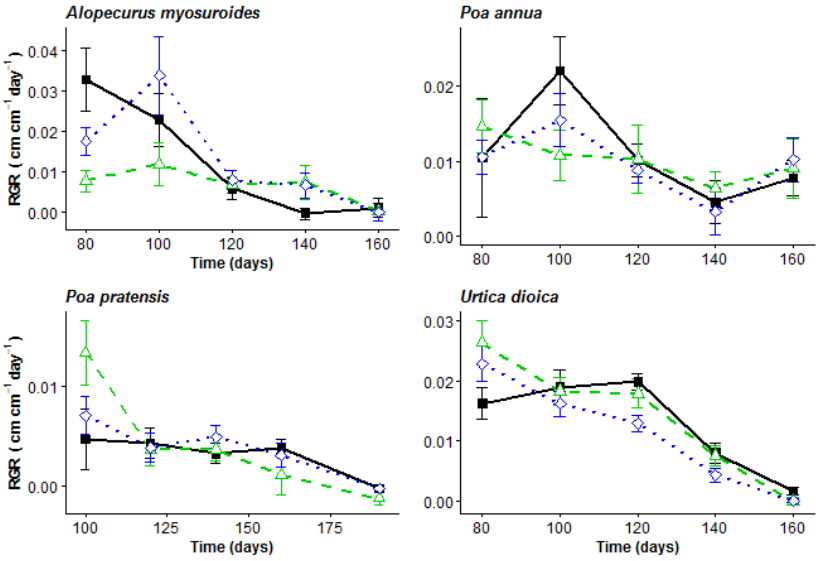

Figure A 4.1 continued.

Table A 4.1 - Results of the non-linear mixed effects models with individual plants as random effects, height as the response variable and dung type as the covariate using a 3 parameter logistic base curve. Asymptote indicates the asymptotic height (φ_1), X_{mid} (φ_2) indicates the time needed to reach half of the asymptotic height and the scale parameter (φ_3) indicates the time elapsed between reaching half and 3/4th of the asymptotic height. P-values are plotted in case dung types differ significantly from the control treatment; 'ns' indicates non-significant differences; '+' and '-' indicate whether the value of the parameter is respectively higher or lower than the value of the control treatment.

species	dung type	asymptote (φ_1)	X_{mid} (φ_2)	scale (φ_3)
Agrostis capillaris	cattle	p=0.002 $^{+}$	ns	p=0.012 $^{-}$
	horse	ns	ns	p=0.038 $^{-}$
Agrostis stolonifera	cattle	p=0.017 $^{+}$	ns	ns
	horse	ns	ns	ns
Alopecurus myosuroides	cattle	ns	ns	ns
	horse	ns	ns	ns
Helianthemum nummularium	cattle	p=0.001 $^{+}$	p<0.001 $^{+}$	ns
	horse	ns	ns	ns
Juncus bufonius	horse	ns	p<0.001 $^{+}$	p=0.035 $^{+}$
Juncus effusus	cattle	ns	p<0.001 $^{+}$	ns
	horse	ns	p=0.012 $^{+}$	ns
Poa annua	cattle	ns	ns	ns
	horse	ns	ns	ns
Poa pratensis	cattle	ns	ns	ns
	horse	ns	ns	ns
Trifolium pratense	cattle	p=0.013 $^{+}$	ns	ns
	horse	ns	ns	ns
Trifolium repens	cattle	ns	ns	ns
	horse	p=0.004 $^{+}$	ns	ns
Tuberaria guttata	cattle	ns	ns	ns
	horse	ns	ns	ns
Urtica dioica	cattle	ns	ns	p=0.016 $^{-}$
	horse	ns	p=0.007 $^{-}$	p=0.036 $^{-}$

Table A 4.2 - Repeated measures ANOVA results in order to evaluate the effect of dung on relative growth rate (RGR) and ramification over time for each of the studied species.

			RGR		ramification	
species	**factor**	***df***	***F* value**	***p***	***F* value**	***p***
Agrostis capillaris	dung	2	1.56	0.22	3.665	0.033
	time	1	616.451	<0.001	1364.535	<0.001
	dung x time	2	7.363	0.001	11.106	<0.001
Agrostis stolonifera	dung	2	0.42	0.663	3.685	0.043
	time	1	159.659	<0.001	81.821	<0.001
	dung x time	2	4.545	0.013	2.815	0.064
Alopecurus myosuroides	dung	2	2.223	0.123	0.912	0.411
	time	1	98.877	<0.001	102.418	<0.001
	dung x time	2	2.322	0.102	13.987	<0.001
Helianthemum nummularium	dung	2	6.812	0.003	0.481	0.621
	time	1	296.354	<0.001	485.22	<0.001
	dung x time	2	1.705	0.184	10.755	<0.001
Juncus bufonius	dung	1	45.924	<0.001	5.854	0.029
	time	1	7.989	0.007	156.775	<0.001
	dung x time	1	2.806	0.101	1.131	0.292
Juncus effusus	dung	2	2.831	0.068	0.005	0.995
	time	1	109.147	<0.001	975.15	<0.001
	dung x time	2	0.327	0.721	7.082	0.001
Poa annua	dung	2	0.213	0.809	1.383	0.261
	time	1	18.856	<0.001	391.065	<0.001
	dung x time	2	0.001	0.999	0.946	0.39
Poa pratensis	dung	2	0.682	0.511	0.641	0.532
	time	1	53.014	<0.001	421.849	<0.001
	dung x time	2	2.341	0.01	9.927	<0.001
Trifolium pratense	dung	2	0.947	0.395	2.436	0.099
	time	1	36.853	<0.001	2050.245	<0.001
	dung x time	2	0.297	0.743	4.088	0.018
Trifolium repens	dung	2	0.699	0.502	3.037	0.057
	time	1	357.961	<0.001	627.6	<0.001
	dung x time	2	2.212	0.112	7.7	<0.001
Tuberaria guttata	dung	2	0.685	0.511	1.323	0.278
	time	1	81.881	<0.001	195.878	<0.001
	dung x time	2	0.516	0.598	2.775	0.065
Urtica dioica	dung	2	2.13	0.127	0.208	0.813
	time	1	205.351	<0.001	765.592	<0.001
	dung x time	2	3.02	0.051	2.506	0.083

5 The impact of dung on inter- and intraspecific competition of endozoochorously dispersed seeds

Tanja Milotić and Maurice Hoffmann

Plants growing in the interspecific competition experiment.

Under review, Journal of Vegetation Science

Abstract

In temperate grasslands, seeds of numerous dry-fruited plant species are dispersed via ingestion and subsequent defecation by grazing animals. Depending on the herbivore species and season, dung pats may contain a large assemblage of conspecific or heterospecific seeds competing for space, light and nutrients in the space-limited environment of an individual dung pat. In an environment rich in nutrients, such as herbivore dung, the outcome of inter- and intraspecific competition might differ from situations where nutrients are limiting. Additionally, dung pats being small and spatially isolated habitats with very specific conditions may impact competitive interactions as well. Complex interactions might, therefore, exist between different seed densities, species combinations and the quality and structure of the dung pat.

We conducted a greenhouse competition experiment using three common perennial grassland species in a replacement design. *Agrostis stolonifera*, *Trifolium pratense* and *Trifolium repens* were used in two-species combinations with different proportions of each species and in monocultures. Seeds were sown in three seed densities (50, 150 and 250 seeds) and the effects of cattle and horse dung on establishment, growth and flowering were tested.

Interactions, most probably attributable to interspecific competition, differed between species mixtures. Seeds sown in polycultures generally germinated faster, but the resulting seedlings had lower relative growth rates compared with seeds sown in monocultures. Increased biomass was measured for each species when growing in polycultures while evidence for intraspecific competition was found in monocultures. *T. pratense* developed relatively more flowers when plants were growing in polycultures compared with monocultures. Few effects of seed densities were found, although higher seed densities led to lower establishment success in both monocultures and polycultures. Adding dung generally increased the time to first germination, relative growth rates and flowering, but decreased establishment success in monocultures.

Both seed density and the presence of dung shape the post-dispersal fate of seeds. While high seed densities imply a cost due to lower germinability, the

nutritive environment of dung acts as a compensation resulting in faster growth and an increased investment in reproductive tissues.

Keywords: dispersal, dispersal success, dung, endozoochory, grassland species, interspecific competition, intraspecific competition, plant competition, post-dispersal seed fate

Introduction

Seed dispersal in general, and specifically endozoochory, has the potential to be advantageous to plants (1) by reducing early seed and seedling mortality near the mother plant caused by density-dependent factors ('escape hypothesis') (Connell, 1971; Janzen, 1970); (2) by facilitating the establishment of new populations ('colonization hypothesis') (Cain et al., 2000; Howe and Smallwood, 1982); and (3) by transporting seeds towards micro sites characterized by the specific narrow range of environmental conditions which favour establishment ('directed dispersal hypothesis') (Cavallero et al., 2012; D'hondt et al., 2012; Wenny, 2001). In recent decades, numerous studies measured the endozoochorous dispersal potential of a wide array of dry-fruited grassland species (e.g., Bakker and Olff (2003), Cosyns et al. (2005a), D'hondt and Hoffmann (2011), Malo and Suarez (1995b), Pakeman et al. (1999), Pakeman et al. (2002)), but few studies examine the next steps in plant establishment. The decisive step in seed dispersal depends on the post-dispersal success of seeds and includes the processes of secondary seed dispersal, seed predation, germination, establishment, growth and the outcome of competitive processes (Nathan and Muller-Landau, 2000; Traveset et al., 2001; Wang and Smith, 2002).

Seeds dispersed through endozoochory end up being deposited in a dung environment which can alter germination success and the subsequent establishment, growth and flowering (Milotić and Hoffmann, 2016b; Ramos-Font et al., 2015). Fresh dung contains high concentrations of growth promoting macronutrients (mainly N and K, and depending on herbivore species lower levels of P, Ca and Mg) and essential trace minerals (mainly

Fe, Mn and Cu) (Haynes and Williams, 1993; Lupwayi et al., 2000; Traveset et al., 2001). However, it must be noted that only a fraction of this nutrient load is inorganic and readily available for plant uptake (Jørgensen and Jensen, 1997). Furthermore, both the dung C: N: P stoichiometry and the rates of dung decomposition and nutrient release are closely related with herbivore diet (browsers vs. grazers) and digestive physiology (ruminants vs. non-ruminants) (Sitters et al., 2014). Factors such as humidity (Dickinson and Craig, 1990; Dickinson et al., 1981), presence of dung fauna (Lovell and Jarvis, 1996; Sitters et al., 2014), chemical composition of dung (Ouédraogo et al., 2004) and vegetation type at the deposition site (Shepherd et al., 2000) define the rate at which dung mineralizes and becomes available for plant uptake in natural ecosystems. Besides the high nutrient content of dung, low concentrations of phytotoxic compounds, such as phenols and fatty acids, have been measured in fresh dung which may alter the activity of enzymes that regulate germination rate and seedling growth in certain plant species (Berendse and Möller, 2009; Farnsworth, 2008; Luna and Moreno, 2009; Marambe et al., 1993). The specific moist conditions within dung pats are thought to have a considerable impact on the early life stages of plants. The moisture content of dung pats is closely related to the decomposition stage and weather conditions (Dickinson et al., 1981) and may even greatly differ between various parts of the dung pat (Underhay and Dickinson, 1978). As the concentration of inorganic nutrients increases and the phytotoxic compounds gradually disappear from decomposing dung, it is expected that the nutritive environment of the dung pat is most useful in the growth phase of plants. Furthermore, as most seeds contain a reserve of mineral and organic nutrients to nourish the embryo in its initial stages of establishment (Fenner and Thompson, 2005), the poor nutrient availability of fresh dung is expected to be of minor importance in the first life stages of plants. In addition to the effects of dung, precipitation may play a significant role in germination and seedling growth (Tjelele et al., 2015).

Dung pats often contain a high concentration of germinable seeds belonging to a wide range of species. In temperate dune grasslands, Cosyns et al. (2005a) for example found on average 463 seedlings per litre of cattle or horse dung (summer sampling) belonging to a total of 31 species. Seed density clearly varies with seed availability in the environment, herbivore type and size, and season (Cosyns et al., 2005a; Malo and Suárez, 1995b)

and a high variability of seed concentrations and species combinations have been found in dung. Seed dispersers may therefore not only affect plant fitness by the act of dispersal itself, but by the different combinations and densities they deposit seeds in dung (Loiselle, 1990).

Despite the presumption that post-dispersal competition for resources modifies the establishment success of seedlings, post-dispersal competition is rarely quantified in endozoochory studies. As competing plants deprive each other of resources such as space, light, water and nutrients, germination in an environment rich in nutrients, such as herbivore dung, might alter the outcome of the competition process compared to a situation where nutrients are limiting. Furthermore, complex interactions might exist between different seed densities, species combinations and the quality and structure of dung. Gu et al. (2012) found negative complementary effects using mixtures of grassland species, and these interspecific competition effects were enhanced with increasing levels of artificial fertilisation. High densities of seeds of the same species may also have an effect on germination timing. In a competition experiment using different seed densities of the same shrub species, Orrock and Christopher (2010) concluded that seeds needed less time to germinate and the resulting seedlings had faster growth rates in highly competitive environments while the germination probability was kept unchanged. Due to the specific environmental conditions in dung pats, they can be considered as safe sites for the germination and establishment of endozoochorously dispersed seeds. As such they might trigger a shift in species co-existing patterns which may locally result in an increase of small-scale species richness (Cosyns et al., 2006). Furthermore, the specific dietary preferences and habitat use of the dispersing herbivore increases the probability of being dispersed to suitable habitats for certain plant species assemblages (Wenny, 2001).

To contribute to improved understanding of at least some of these very diverse aspects of zoochorous dispersal, we conducted a competition experiment using three perennial grassland species commonly found in temperate grasslands which are comparable in seed size (see Bruun and Poschlod (2006) and D'hondt and Hoffmann (2011)). In an attempt to disentangle the effects of different species combinations and seed densities in the presence of dung with the effects of digestive processes in the gut, undigested seeds were used in an experiment in a controlled greenhouse environment. Two clover species (*Trifolium pratense* and *Trifolium repens*)

with short germination times (with mean germination times of respectively 1.9±0.2 and 1.5±0.0 days measured for seeds of the same seed batch in Milotić and Hoffmann (2016c)) and one grass species (*Agrostis stolonifera*) with moderate germination times (with a mean germination time of 10.1±1.8 days in Milotić and Hoffmann (2016c)) were used in two-species combinations with different proportions of each species and in monocultures. Three seed densities were used to assess the effects of different competition levels on the establishment, growth, flowering and biomass of the experimental species. We studied the effects of adding dung of cattle and horses; two herbivore species with a different digestion strategy (respectively ruminants and hindgut fermenters) and which are commonly used in temperate European nature management.

In this study we seek an answer to the following questions:

1. How do inter- and intraspecific competition processes shape the post-dispersal seed fate of different combinations of two early germinating legumes and one slower germinating grass species?
2. To what extent are these competitive processes affected by seed density over a gradient of low, medium and high seed density?
3. How does the dung of two herbivores with distinct digestive strategies (hindgut fermenter vs. ruminant) affect seedling establishment and growth?

Materials and methods

Selected species and seed quality test

Three perennial species were used as model species in the experiments: *Agrostis stolonifera* L., *Trifolium pratense* L. and *Trifolium repens* L. All selected species are common in temperate European grasslands and have been found germinating from dung samples (e.g., Cosyns et al. (2005a)) and were able to germinate in controlled feeding trials (e.g., Cosyns et al. (2005b), D'hondt and Hoffmann (2011)). Seeds were purchased from a webshop specialising in seeds of wild plants (www.herbiseed.com). Although seeds sampled in their natural habitat would give a better

representation, sampling such large quantities of wild seeds was practically unfeasible. By choosing commercial seeds grown during the same year in a temperate climate (Berkshire, UK), seeds of a high and homogeneous quality were used in the experiments, although eventual maternal effects were not completely ruled out using this approach. Seed quality was tested in a standardised laboratory environment by conducting a germination trial over 60 days. For each species, 5 replicates were used and per replicate 50 seeds were sown in Petri dishes filled with 1% water agar substrate. Newly germinated seeds were counted daily during the first 30 days while the counting effort was lowered to once every two days in the last part of the trial. Mean time to germination (MTG) (Ranal and Garcia De Santana, 2006) and the proportion of germinated seeds (germinability) were derived from the count data.

Experimental design

The establishment, biomass, growth and flowering of the selected species were assessed in three dung treatments: cattle and horse dung and a dung-free control treatment. Furthermore, different densities were used with a total seed density of 50 (low density), 150 (medium density) and 250 seeds (high density). Furthermore, seeds were sown in different combinations of monocultures and polycultures in order to assess different levels of inter and intraspecific competition (Figure 5.1). Experimental seed densities were derived from the densities of seeds found germinating in cattle and horse dung in previous research conducted in temperate dune grasslands (Cosyns, 2004). Polycultures consisted of 2 species which were sown in either equal (50%-50%) or unequal (20%-80% or 80%-20%) proportions of the total seed density of 50, 150 or 250 seeds per replicate. All possible combinations of species mixtures, dung treatments and seed densities were made (Appendix, Table A 5.1).

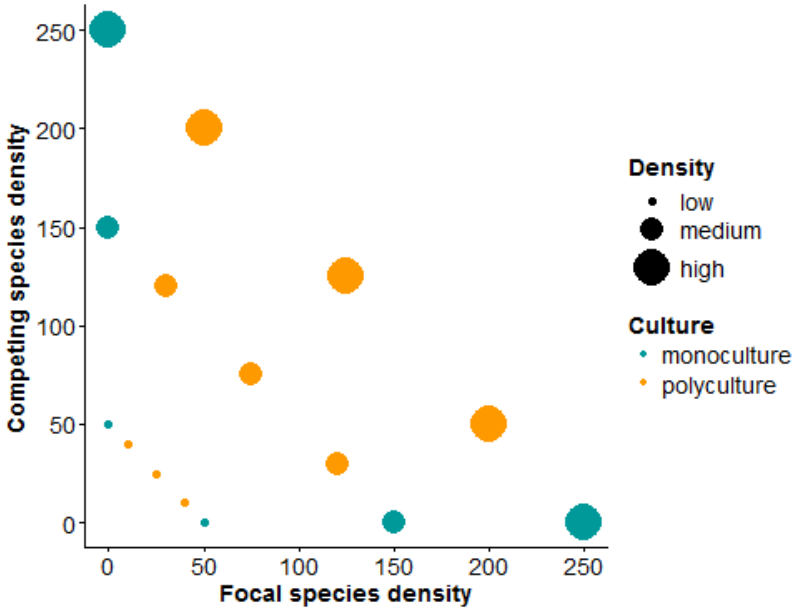

Figure 5.1 - Design of the competition experiment with different densities and combinations of focal species and competing species.

Round plastic plant pots (diameter: 15 cm, height: 16 cm) with drainage holes were filled with a 1:1 mixture of sand and compost. The compost was homogenised and mixed before usage and contained nutrients for the first 6-8 weeks of plant growth (1.25 kg/m³ with N-P-K concentrations of 14-16-18) according to the manufacturer (www.structural.be). Planting pots were put in an unheated greenhouse on felt fabric in order to limit evaporation. Temperature and relative humidity were measured during the experiment using HOBO U23 v2 loggers (ONSET, USA). The mean minimum and maximum temperature and relative humidity respectively were 15.2 °C and 24.7 °C, and 79.6 % and 96.7 %. In order to reduce the influence of the specific micro-climate at the edges, plastic rings (diameter: 9 cm, height: 5 cm) were inserted in the centre of the plant pots. In each ring, intact seeds were sown either mixed with cattle or horse dung or on top of the substrate in the control treatment (Figure 5.2). Furthermore, seeds were sown in one of the three pre-defined seed densities of 50, 150 or 250 seeds and in different species combinations. Each combination of dung treatments, seed densities and species mixtures was replicated five times for each of the

three species with a total of 540 pots in the entire experiment. Fresh dung was collected from stabled cattle and horses fed on a similar diet of pellet feed and hay in order to keep contamination with wild seeds minimal. The sampled dung was mixed prior to application in order to obtain average cattle and horse dung samples. In the dung treatments, rings were filled with 2 cm of either cattle or horse dung after which the seeds were evenly mixed with the dung layer, while in the control treatment seeds were put on top of the substrate. In addition to these control pots which served as a measure for the effects of dung on plant establishment and growth and contained seeds, blank pots were installed as a quality control measure for the used dung and substrate. These blank pots had either a top layer of dung or bare substrate and the emergence of any seedlings was monitored as a control for seed contamination originating from the sand-compost mix, dung or surroundings.

Experimental and blank pots were put in a complete randomised design in order to minimise the impact of location-based environmental fluctuations. Plant pots were watered manually on a daily basis and no artificial light was used. The experiment started on May 30th 2012 when all species were sown and ended after 100 days. Germination timing was monitored by counting emerged seedling every two days during the first two weeks of the experiment and weekly in the following four weeks. Height and flowering (if applicable) were measured after 40, 70 and 100 days. The height of the tallest individual of each species per pot was measured by lifting up the hanging parts or tillers. Flowering was expressed as the sum of the number of flowering and fruiting flower heads of all plants of each species present in each pot. After 100 days, plant establishment was measured by counting the number of plants present at that moment in each pot. A distinction was made between juvenile plants, and flowering and non-flowering plants as well as the visible remains of plants that once established and died during the experiment. All living plants were harvested and separated into below- and above-ground parts. Harvested plants were dried to a constant weight at 65 °C and biomass was measured.

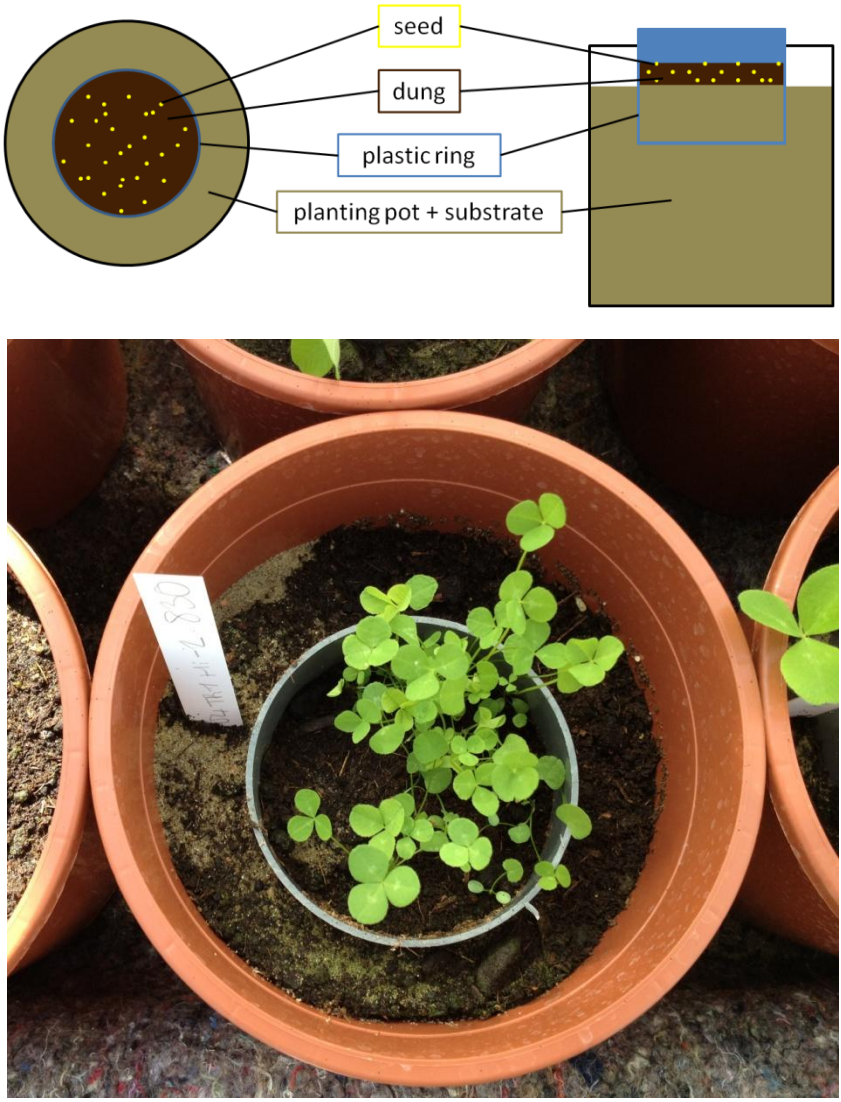

Figure 5.2 - Schematic top view and cross section (respectively top left and top right) and picture (bottom) of the experimental set-up.

Data analysis

Germination timing was analysed using a Cox proportional hazard model with dung type, seed density, competing species and a competing species: proportion interaction term as covariates.

In each census interval, relative growth rates (RGR) were derived from the height of the tallest individual of each species per pot:

$$RGR = \frac{\ln(H_t) - \ln(H_{t-\Delta t})}{\Delta t}$$

where H_t is the maximum height at time t, $H_{t-\Delta t}$ is the height in the previous interval and Δt is the length of the time interval (Hunt, 1982). The effects of dung types and seed densities on RGR were assessed using repeated measures ANOVAs with a proportion: competing species interaction term. RGR was $\log_{10}$ transformed in order to meet the assumptions for parametric tests and Tukey posthoc tests were used to unravel significantly differing dung types and seed densities.

Establishment success in monoculture pots was expressed as the percentage of the sown seeds that established to juvenile plants and flowering or non-flowering adults at the end of the 100-day experiment. Two-way ANOVAs were used to analyse the effects of dung addition and seed densities for each tested plant species. If needed to meet the assumptions for parametric tests, establishment success was $\log_{10}$ or arcsine square root transformed. Significantly differing dung types and seed densities were identified using Tukey posthoc tests.

Competition between species in polycultures was expressed as relative neighbour effect (RNE) (Markham and Chanway, 1996). Two sets of RNE values were calculated using relative establishment success or individual biomass in polycultures and monocultures as follows:

$$RNE = \frac{(M_{mono} - M_{poly})}{x}$$

with M_{mono}: mean establishment success or harvested biomass in monocultures, M_{poly}: mean establishment success or harvested biomass in polycultures and $x=M_{mono}$ if $M_{mono}>P_{poly}$; $x=M_{poly}$ if $M_{poly}>M_{mono}$

RNE values range between -1 and 1, with positive values indicating interspecific competition and negative values indicating facilitation between species (Weigelt and Jolliffe, 2003). Differences in RNE between dung types, seed densities and competing species were assessed with ANOVAs with a proportion: competing species interaction term. *t*-tests were used to identify whether RNE differed significantly from 0.

The bivariate relationships between root and shoot biomass and between the average biomass of individual plants and flower counts were analysed using standardised major axis regressions (SMA) with plant species, dung types, competing species, seed densities and proportions as fixed factors. SMA is an alternative approach to classical regression techniques and is useful in situations where both the *x* and *y* dimensions are subjected to measurement errors (Warton et al., 2006). In a first step, data were tested for common slopes between species, dung types or seed densities. If the slopes did not differ, we tested for common elevations (or *y*-intercepts) between dung types as the lines fitted to the dung types may both represent a shift along their common slope and/or a shift in elevation (Warton et al., 2012). Models were tested for normality and residual distribution and if needed, data were $\log_{10}$ transformed.

All analyses were performed using R version 3.3.1. (R Core Team, 2016b). The 'survival' package version 2.38-3 (Therneau, 2015) and the 'smatr' package version 3.4-3 (Warton et al., 2012) were used for respectively survival analyses and standardised major axis regressions.

Results

In the seed quality test interspecific differences in germination timing were found ($F_{2,12}$= 95.624, p<0.001) as *Agrostis stolonifera* (MTG (se): 10.1 (1.8) d) needed more time compared to *Trifolium pratense* and *Trifolium repens* (MTG (se): respectively 1.9 (0.2) and 1.5 (0.0) d). Also, the proportion of germinated seeds after 60 days clearly differed between species ($F_{2,12}$: 191.001, p<0.001) with the lowest germinability for *A. stolonifera* (22.0 (3.6) %) while nearly all *T. repens* seeds germinated (97.2 (0.4) %) and 88.8 (2.6) % of the seeds of *T. pratense*.

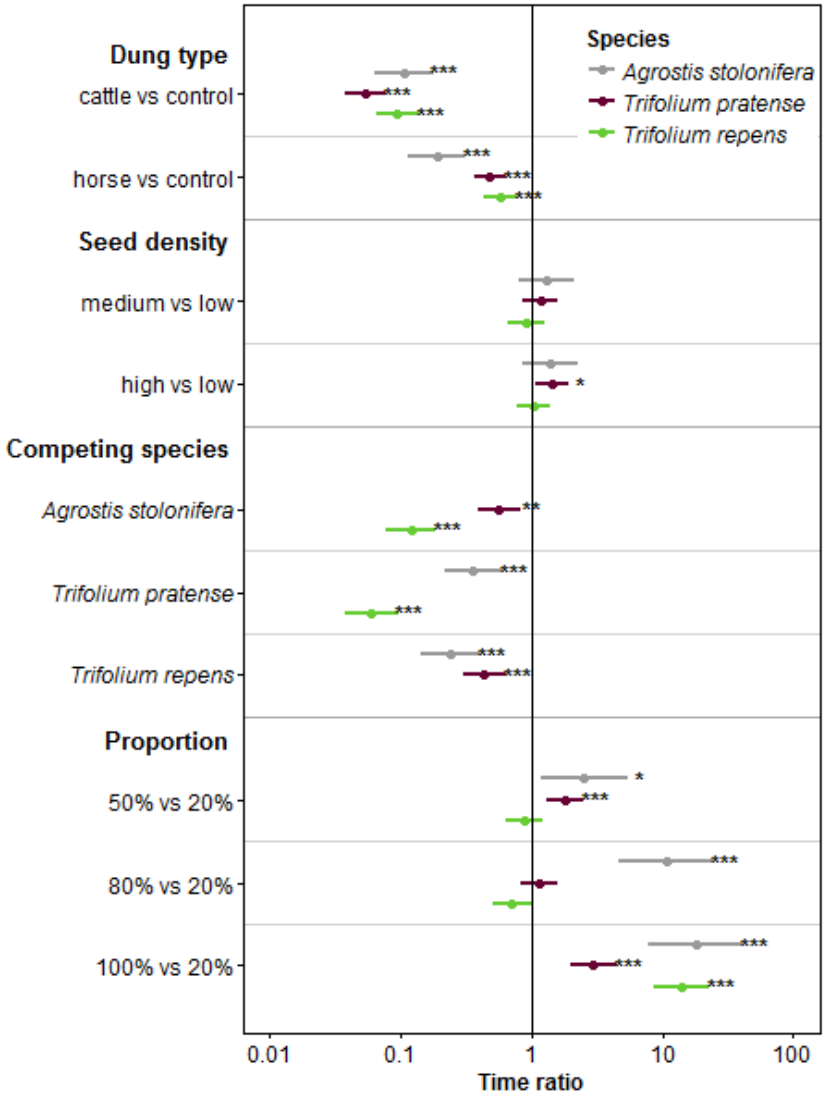

Figure 5.3 - Time ratio (TR) and standard errors by species comparing the germination timing among dung types, seed densities, competing species combinations and seed proportions. TR>1 indicates accelerated germination, while TR<1 represents decelerated germination. Asterisks indicate significance levels found in the Cox proportional hazard model with ***: p<0.001, **: 0.001<p<0.010 and *: 0.010<p<0.050.

In the greenhouse experiments, all tested species needed more time to germinate when sown in dung (z=-5.087, p<0.001) (Figure 5.3). Seed densities generally did not affect germination timing (z=-0.370, p= 0.711), although *T. pratense* germinated slightly faster in high densities compared to low seed density (z= 2.403, p= 0.016). All species had longer germination times when sown in polycultures (z= -2.329, p= 0.020) and in most cases, faster germination was recorded in case seeds were dominant in a mixture (z= 6.158, p<0.001). No seedlings emerged in the blank pots testing for seed contamination originating from dung or substrate.

Table 5.1 - Repeated measures ANOVA results in order to evaluate the effect of dung, seed density, competing species and species proportion on relative growth rate (RGR).

species	factor	*df*	*F* value	*p*
Agrostis stolonifera	dung	2	11.704	<0.001
	seed density	2	0.488	0.616
	time	1	4.591	0.036
	competing species	2	19.785	<0.001
	proportion	4	0.803	0.600
	dung x seed density	4	0.697	0.040
	dung x time	2	3.374	0.345
	density x time	2	1.081	0.933
Trifolium pratense	dung	2	26.631	<0.001
	seed density	2	1.115	0.330
	time	1	0.007	0.933
	competing species	2	193.693	<0.001
	proportion	4	1.084	0.365
	dung x seed density	4	2.566	0.039
	dung x time	2	11.952	<0.001
	density x time	1	10.061	0.002
Trifolium repens	dung	2	15.148	<0.001
	seed density	2	0.162	0.851
	time	1	9.910	0.002
	competing species	2	24.325	<0.001
	proportion	4	0.489	0.744
	dung x seed density	4	0.679	0.607
	dung x time	1	8.058	0.005

For all tested species relative growth rate (RGR) was higher when seeds were sown in cattle or horse dung compared to the control treatment. *Trifolium pratense* and *T. repens* grew respectively 3 and 1.5 times faster when sown in cattle dung compared to horse dung ($F_{2,256}$= 26.631, p<0.001 and $F_{2,238}$= 15.148, p<0.001 respectively). RGR of *A. stolonifera* and *T. repens* decreased significantly over time. While seed densities and different species combinations did not differ in RGR, monocultures consistently had a higher RGR compared to polycultures irrespective of the species that were competing (Table 5.1).

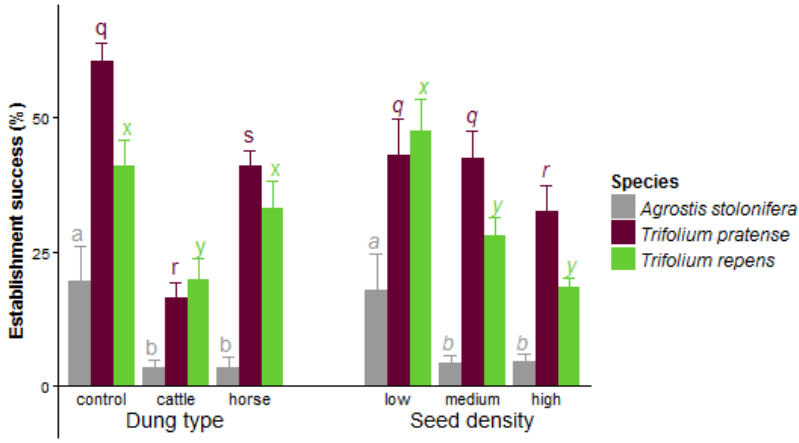

Figure 5.4 - Average establishment success in monocultures by dung type and seed density. Establishment success is expressed as the percentage of sown seeds that grew into plants by the end of the experiment. Error flags indicate standard errors. Letters indicate significant differences between dung types or seed densities for each size class after applying two-way ANOVAs and Tukey posthoc tests. See Appendix, Table A 5.2 for full ANOVA results.

In monocultures, the presence of dung resulted in lower establishment success, especially when seeds were sown in cattle dung (both *Trifolium* species) and overall establishment success decreased with increasing seed densities (Figure 5.4, Appendix, Table A 5.2). The three tested species differed in establishment success as significantly more seeds of the *Trifolium* species emerged compared to *A. stolonifera* ($F_{2,132}$: 40.624; p<0.001). Establishment success of *A. stolonifera* and *T. pratense* in polycultures was altered by interspecific competition effects while the establishment of *T.*

repens was not affected by the presence of competing species when sown in 50%-50% combinations with *A. stolonifera* (Figure 5.5). Relative neighbour effects were affected by the presence of dung, seed densities and competing species (Appendix, Table A 5.3). More specifically, individual plant biomass was relatively higher in polycultures for all tested species. Furthermore, in most cases, differences between species combinations were found (Figure 5.6, Appendix, Table A 5.3). In *A. stolonifera* and *T. pratense* intraspecific competition was stronger when seeds were sown in horse dung compared to the control and cattle dung treatments while dung addition did not have any effect on *T. repens* RNE (Appendix, Table A 5.3).

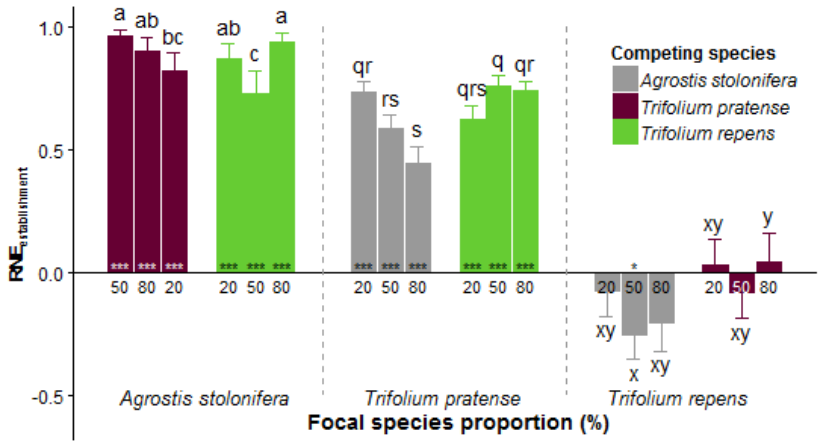

Figure 5.5 - Average relative neighbour effect (RNE) based on the establishment at the end of the experiment for all tested species sown in different proportions with the competing species. Error bars indicate standard errors. Different letters indicate significant differences between species combinations after applying two-way ANOVAs and Tukey posthoc tests (see Appendix, Table A 5.2 for full ANOVA results). Asterisks indicate combinations that differ significantly from 0, with ***: $p<0.001$; **: $0.001<p<0.010$; *: $0.010<p<0.050$ and °: $0.050<p<0.100$.

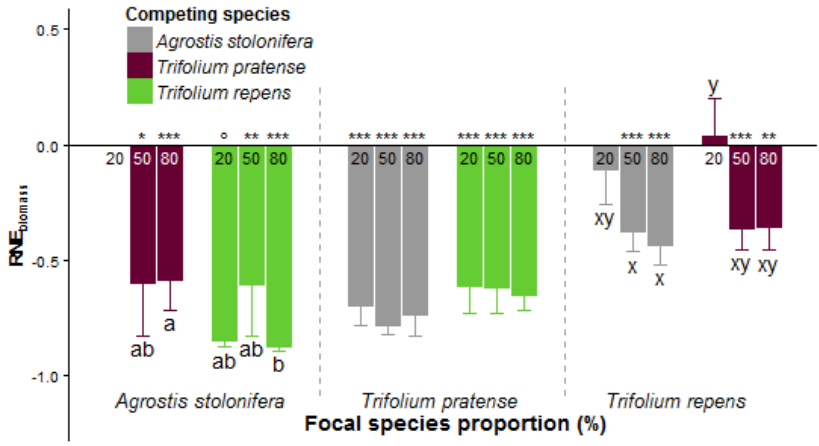

Figure 5.6 - Average relative neighbour effect (RNE) based on individual biomass at the end of the experiment for all tested species sown in different proportions with the competing species. Error bars indicate standard errors. Different letters indicate significant differences between species combinations after applying two-way ANOVAs and Tukey posthoc tests (see Appendix, Table A 5.2 for full ANOVA results). Asterisks indicate combinations that differ significantly from 0, with ***: $p<0.001$; **: $0.001<p<0.010$; *: $0.010<p<0.050$ and °: $0.050<p<0.100$.

Species generally differed in root: shoot biomass allocation and *T. repens* invested more in aboveground biomass (Figure 5.7, Appendix, Table A 5.4 and Table A 5.5). In the experimental treatments, the root: shoot ratio of *A. stolonifera* did not differ among dung types, seed densities or competing species combinations. The addition of cattle dung resulted in a lower root: shoot ratio in both *Trifolium* species compared to the control treatment. Furthermore, the root: shoot ratio was higher in monocultures compared to polycultures for *T. pratense* and *T. repens*. Proportionally more flowers developed on *A. stolonifera* in monocultures than in combination with *T. pratense* while the opposite effect was found for *T. pratense* as significantly more flowers appeared in polycultures compared to monocultures. Plants grown in cattle (both *Trifolium* species) and horse (*T. repens*) dung developed more flowers compared to the control treatment and relatively fewer flowers appeared in high densities of *T. pratense* (Figure 5.8, Appendix, Table A 5.6).

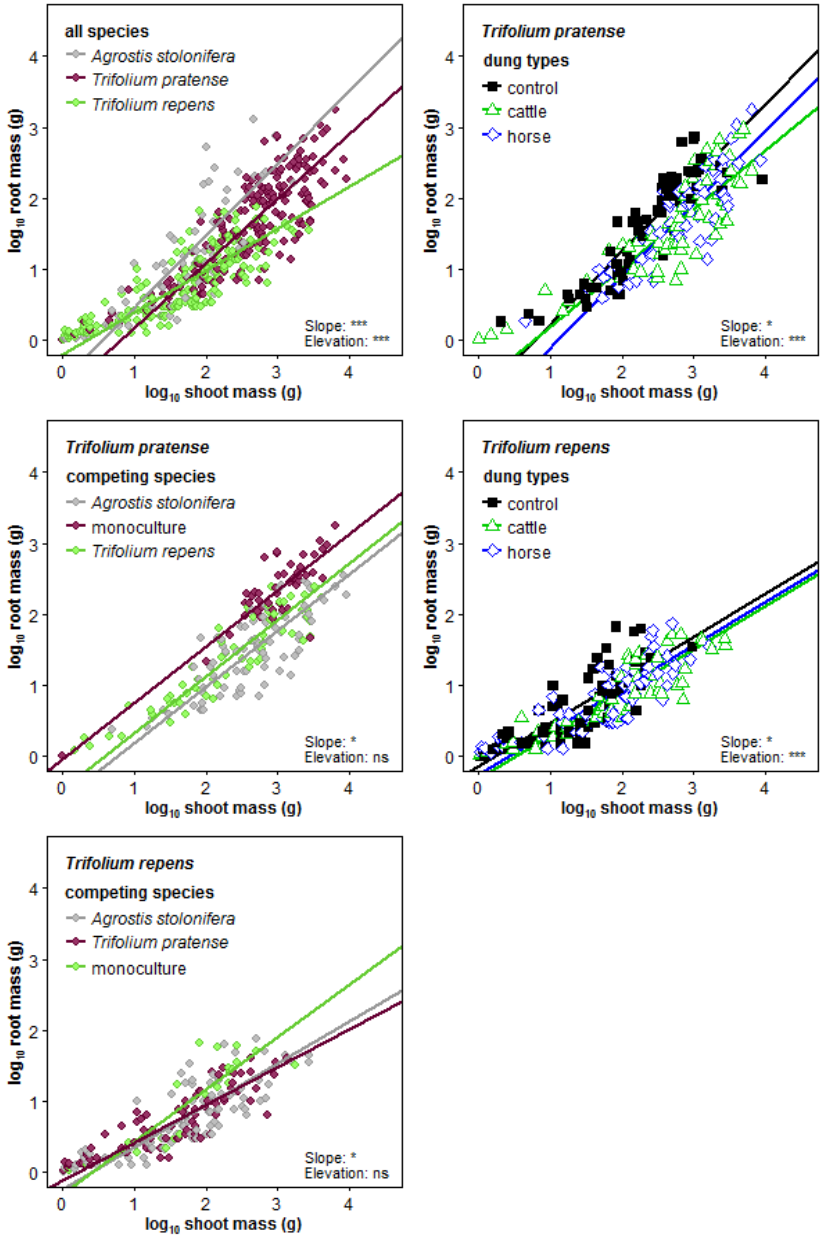

Figure 5.7 - Bivariate plots of standardized major axis regressions (SMA) between root and shoot biomass across dung treatments, seed densities and competing species. Significant differences in SMA elevations (α) and slopes (β) are coded as: ***: $p<0.001$; **: $0.001<p<0.010$; *: $0.050<p<0.010$. Full regression results are provided in Appendix, Table A 5.4 - A 5.6.

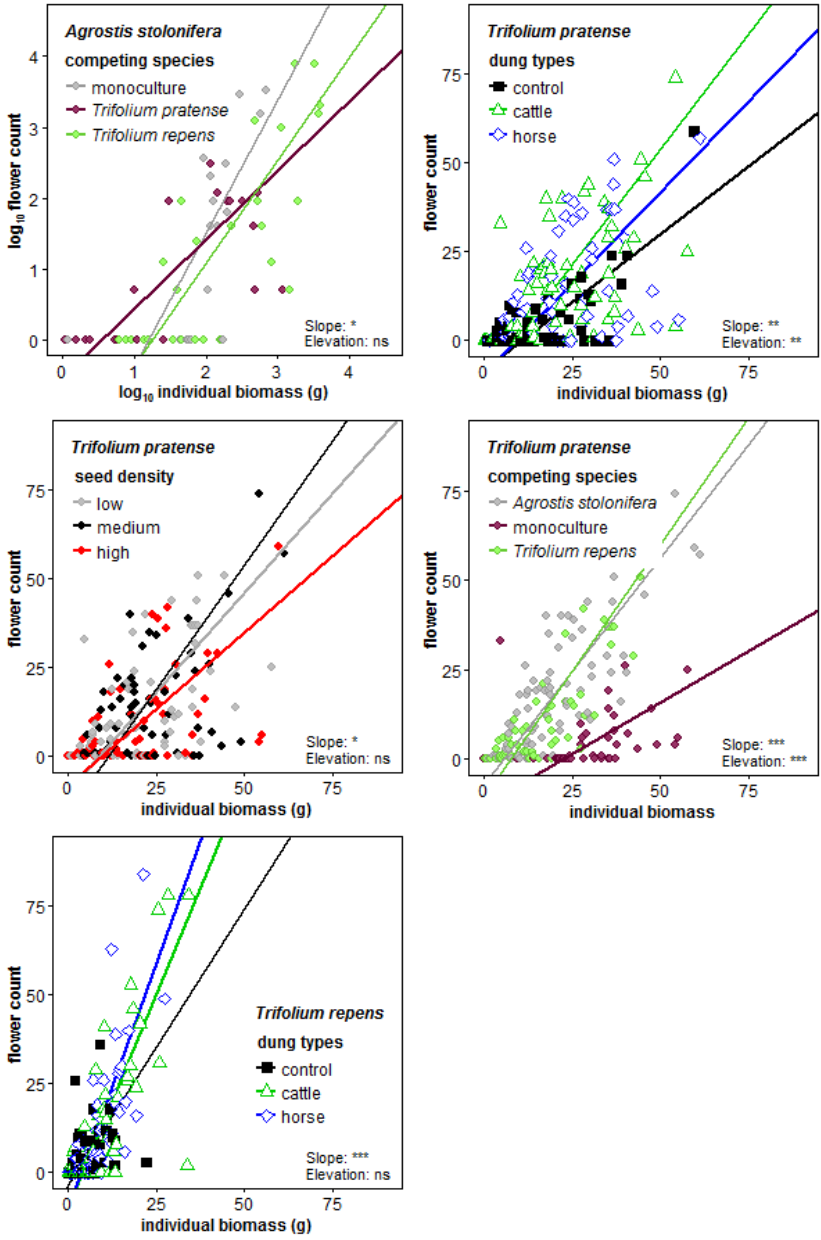

Figure 5.8 - Bivariate plots of standardized major axis regressions (SMA) between flower count and individual biomass (right side), across dung treatments, seed densities and competing species. Significant differences in SMA elevations (α) and slopes (β) are coded as: ***: $p<0.001$; **: $0.001<p<0.010$; *: $0.050<p<0.010$. Full regression results are provided in Appendix, Table A 5.4 - A 5.6.

Discussion

Adding dung generally increased the time to first germination and relative growth rates, and decreased establishment success in monocultures. Seeds sown in polycultures generally germinated faster, but the resulting seedlings had lower relative growth rates. The presence of competing species led to an increase in the biomass and reproductive output of species growing in polycultures while intraspecific competition was found in monocultures. Overall, the presence of dung seems to have a greater impact on plant development compared to the different seed densities that were applied. Germination was delayed and inhibited in our dung treatments, which is in line with the findings of previous research (Meyer and Witmer, 1998; Miller, 1995; Ramos-Font et al., 2015) and has been ascribed to the rather unfavourable environment of fresh dung. Animal excrements may contain toxic compounds with adverse effects on seed survival and germination (Marambe et al., 1993; Welch, 1985). In some cases, dung facilitates fungal and bacterial growth with a mortal effect on seeds (Meyer and Witmer, 1998; Traveset et al., 2007) while in other cases seeds were protected from being attacked by invertebrate predators when covered in a layer of faecal material (Fragoso et al., 2003). On the other hand, once germinated, the growth of young seedlings is in some cases promoted in the dung environment, most probably due to the fertilising effect of this micro-environment (Milotić and Hoffmann, 2016a; Traveset et al., 2001).

Furthermore, both the structural and chemical composition of dung is known to vary between herbivore species (e.g., between ruminants and non-ruminants (Holter, 2016)) or with diet (e.g., brown bear dung composed of animal vs. vegetal material (Traveset et al., 2001)). According to Holter (2016), ruminant dung generally consists of smaller particles and has a higher C: N ratio while the water content is in the same order of magnitude. In our experiment, a hard crust developed soon after application of cattle dung while the appearance of the used horse dung remained structurally unchanged during the whole experimental period. The slower germination rate and lower establishment success of seedlings in cattle dung might be related with this dry and hard top layer (Grellier et al., 2012). The structural composition and water potential of soils are main determinants of plant health and are known to differ greatly between soil types (MacDonald, 1994). Furthermore, plant growth is closely related to the availability of

macro and micro nutrients and toxic elements in the soil environment (Mengel and Kirkby, 1978). Therefore, the differing nutrient concentrations in ruminant and non-ruminant dung could result in a species-specific response in plant development (Dai, 2000; Jørgensen and Jensen, 1997). As fresh dung contains relatively high levels of toxic compounds and most nutrients are bound in organic compounds that need to be mineralized before becoming available for plant uptake, dung might have adverse effects on the early life stages of plants (Cosyns et al., 2006; Ramos-Font et al., 2015). Furthermore, dung may also promote the growth of fungi and bacteria which may damage seeds and inhibit germination and seedling survival (Clark and Wilson, 2003; Traveset et al., 2007).

As high seed densities generally have a negative impact on plant establishment, a selection pressure can be expected for tactics that overcome this competition, e.g., early germination or induced dormancy (Loiselle, 1990; Murray, 1998). Orrock and Christopher (2010) measured shorter germination times when intraspecific competition was greater in a bird-dispersed shrub and early germinated plants grew larger and had higher growth rates as they had more time to grow without competitors. Likewise examples of the advantages of early germination can be found in both intraspecific (e.g., Black and Wilkinson (1963)) and interspecific (e.g., Bergelson and Perry (1989)) seed mixtures while other species might postpone germination until the subsequent season in highly competitive environments (Turkington et al., 2005). In a laboratory experiment, Linhart (1976) measured an increased germination rate in *Trifolium* and *Agrostis* species when sown in high densities. Although we should note that we used different methods and species, we did not measure any effect of seed density on germination timing. Overall, high seed densities led to a reduced establishment success in monocultures which may be attributed to intraspecific competition or to the mechanism of autotoxicity that results in an inverse correlation between seed abundance and germination success (Barnea et al., 1992; Loiselle, 1990; Murray, 1998). By adapting the seed germination - dormancy patterns, plants may be able to escape sibling competition in high densities (Cheplick, 1992; Hyatt and Evans, 1998).

The functional equilibrium theory (Brouwer, 1962) postulates that the allocation of biomass shifts towards roots when belowground resources (e.g., nutrient level and water) are low whereas shoot biomass increases in case aboveground resources (e.g., CO_2 and light) are limiting. Doing so,

plants optimise their ability to compete for limiting resources (Poorter and Nagel, 2000). Our results partly support this theory, as both *Trifolium* species indeed had a higher root biomass in the control treatment. Nevertheless, few interactions between dung treatments and seed densities were found which is in contrast with the differing root: shoot biomass allocation in *Plantago lanceolata* with varying seed densities and nutrient levels found by Berendse and Möller (2009) or the increased belowground competition Aerts et al. (1991) found in fertilised heathland plants.

In all tested species individual plant biomass was relatively higher in polycultures which suggests that intraspecific competition has stronger effects than interspecific competition. Higher biomass of species in polycultures has been previously found in natural ecosystems and increases with increasing nutrient levels (Gu et al., 2012; Li and Watkinson, 2000). On the other hand, lower establishment of *A. stolonifera* and *T. pratense* has been found in polycultures, so rather than producing more biomass spread over a large number of individuals, the average biomass per individual was greater in polyculture stands. Furthermore, both *Trifolium* species invested more in belowground biomass when grown in monocultures compared with polycultures which suggests that intraspecific competition is harsher than interspecific competition in our species mixtures. Furthermore, through a symbiotic relationship with soil bacteria of the genus *Rhizobia* in their root nodules both *Trifolium* species are able to bind atmospheric nitrogen into ammonium and gain a competitive advantage. In grass-clover mixtures, Nesheim and Boller (1991) noted an increased nitrogen fixation when clovers were growing in combination with grasses whereas Ledgard and Steele (1992) measured reduced nitrogen fixation when N fertiliser was used. In our experiments, we indeed measured a higher allocation to *Trifolium* root biomass in the control treatments. The presence of dung following endozoochorous dispersal might, therefore, change the competitive interactions of species mixtures. Furthermore, the reproductive effort decreases with increasing seed density in *T. pratense* which could be related to the fact that individual plants are smaller in high seed densities and can, therefore, allocate a smaller proportion of their biomass in reproductive tissues (Weiner, 1988). Similar effects have been found for the species that grew taller in the dung treatments.

Our results suggest that the presence of dung and competition affects endozoochorous dispersal success. However, we should keep in mind that

we conducted our experiment in a standardised greenhouse environment which differs from the natural situation. As we directly put dung on the substrate we did not account for competition by already established vegetation. Large herbivores deposit a rather large quantity of dung per defecation and their dung often smothers and kills the existing vegetation at the deposition site and consequently creates a gap for seedling establishment (Traveset, 1998; Williams and Haynes, 1995). In a dung deposition experiment in temperate dune grasslands, Cosyns et al. (2006) measured an increase in small-scale species richness as species co-existing patterns shifted from a dominance by monocotylous species towards a more diverse assemblage of dicotylous and monocotylous species. Therefore, dung pats not only seem to trigger inter- and intra-specific competition processes between their seed load, but also create safe sites in the existing vegetation enabling the germination and establishment of endozoochorously dispersed seeds.

Throughout the experiment we used a 2 cm thick layer of dung, which should enable the germination of the rather large seeded test species (see e.g., Grundy et al. (2003)), but in natural situations cattle dung pats are frequently thicker than 2 cm (Malo and Suárez, 1995a) and horses often exhibit latrine behaviour by which both dung and dispersed seeds becomes aggregated (Edwards and Hollis, 1982). Furthermore, due to practical considerations, intact seeds were used while ingested seeds undergo a sequence of mechanical, thermal and chemical processes (Milotić and Hoffmann, 2016b). A decreased germination success has been observed after gut passage in many species (e.g., Cosyns et al. (2005b)). In a gut passage experiment using cattle, D'hondt and Hoffmann (2011) found an increased germination success of *T. pratense* while *A. stolonifera* seeds were less likely to germinate after gut passage. On the other hand, both of our tested *Trifolium* species had a lower germinability after being fed to horses and sheep (Cosyns et al., 2005b). Therefore, our results should rather be interpreted as the outcome of one particular step in the complex process of endozoochory, and we can assume that an even lower germination success would have been found when using ingested seeds.

We conclude that the variable concentrations and species combinations in dung pats may trigger processes of inter- and intraspecific competition and hence define the post-dispersal fate of endozoochorously dispersed seeds. While high seed densities imply a cost due to lower germinability and

establishment, the nutritive environment of dung acts as a compensation resulting in faster growth and an increased investment in reproductive tissues in competitive superior species.

Acknowledgments

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Appendix

Table A 5.1 - Experimental scheme with all treatment combinations of dung types, seed densities and species combinations. Numbers indicate the number of seeds of the focal species (in bold) and competing species (between brackets). Each combination was replicated 5 times.

			focal species		
dung type	**seed density**	**competing species**	*Agrostis stolonifera*	*Trifolium pratense*	*Trifolium repens*
control, cattle or horse	low	*Agrostis stolonifera*	**50** (0)	**10** (40), **25**(25) or **40**(10)	**10** (40), **25**(25) or **40**(10)
		Trifolium pratense	**10** (40), **25**(25) or **40**(10)	**50** (0)	**10** (40), **25**(25) or **40**(10)
		Trifolium repens	**10** (40), **25**(25) or **40**(10)	**10** (40), **25**(25) or **40**(10)	**50** (0)
	medium	*Agrostis stolonifera*	**150** (0)	**30** (120), **75**(75) or **120**(30)	**30** (120), **75**(75) or **120**(30)
		Trifolium pratense	**30** (120), **75**(75) or **120**(30)	**150** (0)	**30** (120), **75**(75) or **120**(30)
		Trifolium repens	**30** (120), **75**(75) or **120**(30)	**30** (120), **75**(75) or **120**(30)	**150** (0)
	high	*Agrostis stolonifera*	**250** (0)	**50** (200), **125**(125) or **200**(50)	**50** (200), **125**(125) or **200**(50)
		Trifolium pratense	**50** (200), **125**(125) or **200**(50)	**250** (0)	**50** (200), **125**(125) or **200**(50)
		Trifolium repens	**50** (200), **125**(125) or **200**(50)	**50** (200), **125**(125) or **200**(50)	**250** (0)

Table A 5.2 - Two-way ANOVA results with establishment success in monocultures as measured variable and dung type and seed density as nominal variables. Establishment success is expressed as the percentage of sown seeds that established to visible plants at the end of the experiment and was analysed for each size class. Significant results are written in bold.

			size class			
species	**factor**	***df***	**all**	**non-flowering**	**flowering**	**juvenile**
Agrostis	dung	2	***F*: 6.649; *p*=0.003**		***F*: 3.291; *p*=0.049**	***F*: 7.605; *p*=0.002**
stolonifera	seed density	2	***F*: 4.604; *p*=0.017**		*F*: 1.270; *p*=0.293	***F*: 3.891; *p*=0.030**
	dung x density	4	*F*: 1.872; *p*=0.137		*F*: 0.498; *p*=0.737	*F*: 1.905; *p*=0.131
Trifolium pratense	dung	2	***F*: 67.459; *p*<0.001**	***F*: 37.002; *p*<0.001**	***F*: 28.221; *p*<0.001**	***F*: 37.444; *p*<0.001**
	seed density	2	***F*: 4.714; *p*=0.015**	***F*: 7.416; *p*=0.002**	***F*: 25.490; *p*<0.001**	***F*: 9.508; *p*<0.001**
	dung x density	4	*F*: 1.542; *p*=0.211	***F*: 3.845; *p*=0.011**	***F*: 12.087; *p*<0.001**	*F*: 1.302; *p*=0.288
Trifolium repens	dung	2	***F*: 10.030; *p*<0.001**		*F*: 0.028; *p*=0.973	*F*: 1.6477; *p*=0.207
	seed density	2	***F*: 12.776; *p*<0.001**		*F*: 1.451; *p*=0.248	*F*: 2.2351; *p*=0.122
	dung x density	4	*F*: 0.946; *p*=0.449		*F*: 0.303; *p*=0.874	*F*: 0.6059; *p*=0.661

Table A 5.3 - ANOVA results with RNE based on establishment success and biomass as measured variable and dung type, seed density and competing species as nominal variables and a nested proportion: competing species term.

		$RNE_{establishment}$			$RNE_{biomass}$		
focal species	**factor**	***df***	***F* value**	***p***	***df***	***F* value**	***p***
Agrostis stolonifera	dung type	2	3.444	0.034	2	3.75	0.034
	seed density	2	115.2	<0.001	2	7.078	0.003
	competing species	1	3.251	0.073	1	4.42	0.043
	proportion	4	8.661	<0.001	3	3.586	0.024
	dung x density	4	110.9	<0.001	4	1.61	0.194
Trifolium pratense	dung type	2	8.727	<0.001	2	27.37	<0.001
	seed density	2	5.536	0.004	2	20.7	<0.001
	competing species	1	9.74	0.002	1	6.36	0.013
	proportion	4	4.689	0.001	4	1.423	0.23
	dung x density	4	2.845	0.025	4	15.3	<0.001
Trifolium repens	dung type	2	55.5	<0.001	2	0.24	0.787
	seed density	2	27.62	<0.001	2	3.309	0.039
	competing species	1	7.591	0.006	1	0.264	0.608
	proportion	4	1.258	0.287	4	2.801	0.028
	dung x density	4	11.836	<0.001	4	5.114	<0.001

Table A 5.4 - Likelihood ratios and p-values for the SMA regressions between root and shoot biomass and between flower count and total biomass by species with dung types, seed densities and competing species as factors.

interaction term	species	factor	likelihood ratio (*df*)	*p*
root: shoot ratio	all	species	72.66 (2)	<0.001
	Agrostis stolonifera	dung types	0.16 (2)	0.923
		seed density	1.44 (2)	0.486
		competing species	4.10 (2)	0.129
	Trifolium pratense	dung types	6.86 (2)	0.032
		seed density	1.47 (2)	0.480
		competing species	100 (2)	<0.001
	Trifolium repens	dung types	11.82 (2)	0.003
		seed density	2.28 (2)	0.319
		competing species	7.74 (2)	0.021
flower count: biomass	*Agrostis stolonifera*	dung types	0.14 (2)	0.932
		seed density	3.05 (2)	0.217
		competing species	7.44 (2)	0.024
	Trifolium pratense	dung types	12.76 (2)	0.002
		seed density	8.88 (2)	0.012
		competing species	26.49 (2)	<0.001
	Trifolium repens	dung types	16.66 (2)	<0.001
		seed density	1.12 (2)	0.572
		competing species	1.79 (2)	0.409

Table A 5.5 - Standardized major axis (SMA) regression coefficients between root and shoot biomass by species and factor. Only factors which differ significantly after a likelihood test are listed (see Table A 5.4). Factor levels marked with different letters differ significantly (p<0.050) after multiple comparisons with Šidák correction.

species, factor	factor level	n	α_{SMA} (95% CI)	β_{SMA} (95% CI)	R^2	p
all		453	-0.47 (-0.55; -0.38)	0.81 (0.77; 0.85)	0.74	<0.001
	Agrostis stolonifera[a]	70	-0.55 (-0.80; -0.32)	1.02 (0.89; 1.17)	0.69	<0.001
	Trifolium pratense[a]	187	-0.73 (-0.93; -0.54)	0.91 (0.84; 0.99)	0.7	<0.001
	Trifolium repens[b]	196	-0.21 (-0.29; -0.13)	0.59 (0.55; 0.64)	0.7	<0.001
Trifolium pratense						
dung types	control[a]	42	-0.82 (-1.10; -0.53)	1.04 (0.93; 1.16)	0.8	<0.001
	cattle[b]	16	-0.64 (-0.96; -0.32)	0.83 (0.73; 0.95)	0.71	<0.001
	horse[a]	12	-1.12 (-1.54; -0.70)	1.02 (0.88; 1.18)	0.72	<0.001
competing species	monoculture[a]	45	-0.05 (-0.24; 0.15)	0.80 (0.74; 0.86)	0.73	<0.001
	Agrostis stolonifera[b]	79	-0.62 (-0.79; -0.45)	0.80 (0.74; 0.86)	0.65	<0.001
	Trifolium repens[c]	63	-0.47 (-0.62; -0.32)	0.80 (0.74; 0.86)	0.83	<0.001
Trifolium repens						
dung types	control[a]	70	-0.14 (-0.23; -0.05)	0.61 (0.56; 0.66)	0.65	<0.001
	cattle[b]	59	-0.31 (-0.43; -0.20)	0.61 (0.56; 0.66)	0.78	<0.001
	horse[b]	67	-0.26 (-0.36; -0.15)	0.61 (0.56; 0.66)	0.69	<0.001
competing species	monoculture[a]	25	-0.32 (-0.61; -0.03)	0.74 (0.60; 0.91)	0.77	<0.001
	Agrostis stolonifera[ab]	88	-0.25 (-0.39; -0.11)	0.59 (0.53; 0.68)	0.66	<0.001
	Trifolium pratense[b]	83	-0.12 (-0.21; -0.02)	0.53 (0.48; 0.59)	0.76	<0.001

Table A 5.6 - Standardized major axis (SMA) regression coefficients between flower counts and total biomass by species and factor. Only factors which differ significantly after a likelihood test are listed (see Table A 5.4). Factor levels marked with different letters differ significantly (p<0.050) after multiple comparisons with Šidák correction.

species, factor	factor level	n	α_{SMA} (95% CI)	β_{SMA} (95% CI)	R^2	p
all		453	-1.49 (-1.74; -1.24)	1.37 (1.27; 1.47)	0.37	<0.001
Agrostis stolonifera						
competing species	monoculture[a]	19	-2.24 (-3.69; -0.79)	1.88 (1.30; 2.72)	0.45	0.002
	Trifolium pratense[b]	22	-0.54 (-1.10; 0.02)	0.98 (0.72; 1.34)	0.54	<0.001
	Trifolium repens[ab]	29	-1.81 (-2.71; -0.91)	1.45 (1.12; 1.88)	0.56	<0.001
Trifolium pratense						
dung types	control[a]	42	-8.04 (-11.25; -4.82)	0.76 (0.63; 0.93)	0.38	<0.001
	cattle[b]	16	-10.93 (-18.71; -3.15)	1.30 (1.05; 1.61)	0.26	<0.001
	horse[ab]	12	-9.16 (-17.18; -1.13)	1.02 (0.79; 1.32)	0.09	0.026
seed density	low[a]	61	-9.68 (-15.98; -3.38)	1.12 (0.90; 1.39)	0.3	<0.001
	medium[a]	63	-15.82 (-24.13; -7.51)	1.40 (1.12; 1.74)	0.26	<0.001
	high[b]	63	-7.91 (-13.25; -2.56)	0.86 (0.69; 1.09)	0.18	<0.001
competing species	monoculture[a]	45	-12.92 (-19.04; -6.79)	0.58 (0.43; 0.78)	0.04	0.186
	Agrostis stolonifera[b]	79	-6.85 (-10.93; -2.78)	1.27 (1.11; 1.45)	0.64	<0.001
	Trifolium repens[b]	63	-10.65 (-15.30; -6.01)	1.42 (1.21; 1.66)	0.62	<0.001
Trifolium repens						
dung types	control[a]	70	-3.51 (-5.91; -1.11)	1.56 (1.25; 1.95)	0.15	0.001
	cattle[b]	59	-9.54 (-15.03; -4.04)	2.40 (2.02; 2.86)	0.56	<0.001
	horse[b]	67	-10.33 (-14.78; -5.89)	2.77 (2.35; 3.27)	0.55	<0.001

6 The relation between herbivore presence and the expansion of *Helianthemum nummularium* in grassland-scrub mosaic vegetation: circumstantial evidence for zoochorous seed dispersal and grazing preference impact

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Flowering *Helianthemum nummularium* (L.) Mill. ©Bastiaan Notebaert

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Abstract

Extensive grazing with large domestic herbivores often has a strong influence on the structure and composition of herbaceous plant communities with increasing population sizes for some grassland plants and decreasing presence for others. Herbivores affect plant communities directly by selective grazing of plant species, habitats and the landscape, and indirectly by transporting seeds through attachment (epizoochory) and ingestion and subsequent defecation (endozoochory). Whether a certain plant species increases or decreases in cover under grazing largely depends on its ability to cope with trampling, defoliation, and dung deposition. Hence, according to their response to herbivore activities, plant species can be classified as grazer increaser or grazer decreaser species. *Helianthemum nummularium* is considered as an increaser species since its distribution increased remarkably after the introduction of free-ranging large grazers in at least two coastal dune grassland areas in Belgium. Nonetheless, its seeds lack any obvious dispersal-related adaptations, and direct observations of plant/seed consumption are scarce.

Through field and laboratory experiments, we assessed the dispersal suitability of *H. nummularium* via endozoochorous and epizoochorous dispersal methods. In a differentiated grazer exclusion experiment, evidence was found that plants are grazed by both large domestic ungulates (cattle or horses) and small wild herbivores (rabbits or hares). Direct endozoochory evidence remains scarce. No seeds were found germinating in field-collected dung, and only few seedlings emerged following a seed feeding experiment. However, once deposited, we found higher growth rates when seeds were mixed with dung, while on the other hand establishment success lowered when seeds were grown in combination with competitively superior species such as *Trifolium pratense*. Epizoochory evidence is stronger: this dispersal mechanism is plausible since both the fur and hooves of cattle and horses were found to potentially contribute to transport of *H. nummularium* seeds although this was hardly the case in high and dense vegetation. From the literature we extracted that large herbivores as cattle and horses are able to maintain open grassland-scrub mosaic dune grassland vegetation, which is generally considered an optimal landscape for *H. nummularium.*

We conclude that the increasing cover of *H. nummularium* after introducing domestic herbivores most probably is the result of a complex interplay

between various ecological processes. Herbivores certainly play a role in the dispersal of *H. nummularium* seeds through internal and external ways while their selective grazing behaviour most probably creates an appropriate environment for *Helianthemum* establishment and maintenance.

Keywords: endozoochory, epizoochory, grazing, *Helianthemum nummularium*, seed dispersal

Introduction

In Western Europe, the conservation of species-diverse, semi-natural landscapes face a multitude of threats. Human activities have resulted in habitat loss and fragmentation which disrupt large-scale ecological and geomorphological processes and inevitably cause the disappearance of many plant and animal species (Wallis De Vries, 1995). Key organisms in these ecosystems have been extinct for centuries, e.g. large herbivores such as aurochs, tarpan or (regionally) European bison. More recently, the traditional and small-scale agropastoral practices, such as extensive grazing, hay making, coppicing and turf cutting, which once led to the creation of semi-natural, species-rich habitats and landscapes, have been replace by more intensive management (Eriksson et al., 2002; Pykälä, 2000; Wallis De Vries, 1995). In order to counteract the deterioration of such semi-natural habitats, many of these traditional agropastoral methods have been reintroduced in nature management, often in highly fragmented, isolated, small-scale nature areas in Europe. Free-ranging domestic herbivores, such as cattle, horses, and sheep, are commonly used in nature management in an attempt to prevent shrub encroachment and to create spatial heterogeneity (Adler et al., 2001; Cosyns and Hoffmann, 2005; Lamoot et al., 2005b; Olff and Ritchie, 1998). Apart from the selective removal of plant biomass and the creation of regeneration sites through trampling, grazers also impact the dispersal of propagules which might be of prime importance in the restoration of plant biodiversity in these fragmented landscapes (Mouissie et al., 2005c; Olff and Ritchie, 1998). For instance, in a large-scale

meta-analysis, Albert et al. (2015a) concluded that at least 44% of the available plant species in grazed ecosystems were (potentially) dispersed by ungulates.

Animal-mediated dispersal implies either the external attachment of diaspores on hooves or fur (epizoochory) or the ingestion and subsequent excretion of germinable seeds in dung (endozoochory). These zoochorous dispersal modes can be considered complementary (Couvreur et al., 2005a) and the likelihood of being dispersed through one of these mechanisms largely depends on habitat, plant and herbivore characteristics, such as habitat openness, diaspore morphology, diaspore release height, shoulder height, hair curliness and length, body mass, diet choice and digestive system (Albert et al., 2015a; Albert et al., 2015b). Many cases of long-distance dispersal of propagules by animals are documented (Cain et al., 2000; Higgins et al., 2003; Nathan et al., 2008). As this process involves a certain level of directedness (D'hondt et al., 2012; Wenny, 2001) zoochorous dispersal could be of prime importance to remain or restore viable plant populations in fragmented landscapes. Furthermore, dispersal mechanisms differ in effectiveness both quantitatively (number of dispersed seeds and dispersal distance) and qualitatively (dispersal environment and seed deposition site) (Schupp et al., 2010). Once viable seeds have been dispersed successfully, their ability to establish and grow into mature plants largely depends on the quality of the deposition site. Endozoochorously dispersed seeds inevitably are excreted in seed clumps with dung that reduces germination success (Milotić and Hoffmann, 2016c), but potentially promotes plant growth and flowering (Milotić and Hoffmann, 2016a). On the other hand, epizoochorously dispersed seeds are deposited in a more scattered pattern and are not directly affected by the presence of dung (Couvreur et al., 2005b).

Introducing large herbivores often has a strong influence on the structure and composition of herbaceous plant communities with increased population sizes for many grassland plants (e.g., Cosyns et al. (2005a), Couvreur et al. (2005a), Poschlod et al. (2011)) while other species decrease (Carmona et al., 2013). Hence, according to their response to herbivore activity, plant species can be classified as grazer increaser or grazer decreaser species (Carmona et al., 2013). Whether a certain plant species increases or decreases its abundance under grazing largely depends on its ability to cope with trampling, defoliation and dung (Dobarro et al., 2013)

and, more in general, grazing pressure (Hobbs and Huenneke, 1992; Olff and Ritchie, 1998). Another herbivore with an unmistakable impact on vegetation composition is the rabbit which was introduced in Western Europe in the 11th-12th century (Provoost et al., 2011b; Thompson and King, 1994). Rabbits alter vegetation structure and plant species composition through grazing and digging (Delibes-Mateos et al., 2008), but they potentially also play a role in the dispersal of seeds through endozoochory (Cosyns et al., 2005b; Pakeman et al., 2002; Pakeman et al., 1999). Finally, their latrines increase soil fertility and create open areas (Delibes-Mateos et al., 2008).

Helianthemum nummularium (L.) Mill is an example of a grazing increaser species as its distribution patterns, age structure and mode of regeneration are affected by grazing management with the highest population densities and more vegetative propagation being found under grazing management (Poschlod et al., 2011). In coastal dune areas in Belgium, its distribution has increased remarkably since the introduction of free-ranging grazers (Provoost et al., 2015; Van Landuyt et al., 2006a). *H. nummularium* is a hemiphanerophyte belonging to the Cistaceae family and is regionally known as a rare to very rare species with red list status in different regions of Europe (Proctor, 1956; Van Landuyt et al., 2006b). While the species has a sub-Mediterranean distribution ranging from the Iberian Peninsula, through central and southern Europe and northwards to Scotland, Denmark, central Sweden and southern Finland with scattered populations in the Caucasus and Russia (Volkova et al., 2016), in Flanders the species is restricted to calcium rich grasslands (Van Landuyt et al., 2006b). The round, smooth and rather small seeds lack any obvious dispersal-related adaptations in dry conditions, but develop a sticky mucilage layer when wet (Baskin and Baskin, 2001). Furthermore, Cistaceae species are known to have a high incidence of physical dormancy (Thanos et al., 1992) and germination is in some cases triggered through endozoochorical dispersal (Jaganathan et al., 2016). Through field and laboratory experiments we assessed the suitability of *H. nummularium* in dispersing through endozoochorous and epizoochorous dispersal methods.

More specifically, we attempt to find an answer to the following research questions:

1. What herbivore characteristics are the most probable contributors to the expansion of *Helianthemum nummularium*: grazing preference, endozoochory or epizoochory?
2. Is *H. nummularium* actively grazed by herbivores? If so, how often, which parts and by which herbivore species?
3. Are *H. nummularium* seeds able to survive ingestion by large herbivores?
4. Once deposited in dung, what is the post-dispersal fate of seeds?

Are the seeds of *H. nummularium* suited for epizoochorous dispersal? By which body parts and what is the role of abiotic conditions?

Materials and methods

Study area

The field experiments were conducted in the Flemish nature reserve 'De Westhoek' which is a temperate coastal dune area located along the Belgian-French border (51°4'45''N; 2°33'40''E). The soil type of the northern part of the reserve where the study took place is an eutric regosol (IUSS Working Group, 2006). The area can be classified as a relatively young parabolic dune landscape with spatially heterogeneous vegetation consisting of mobile yellow dunes, Marram dunes, moss-dominated dunes, moist to dry dune grasslands, wet to moist dune slack vegetation, and relatively young dune scrub (Tahmasebi, 2008). *Helianthemum nummularium* typically grows in moist dune grasslands and along scrub borders in a scrub-grassland mosaic landscape. At the time of the study, the Westhoek reserve was subdivided into two spatially disjunct grazing areas (80 ha in the north and 60 ha in the south) and a large ungrazed area. The largest population of *H. nummularium* is situated in the northern part which has been grazed by a fluctuating number of free-ranging Highland cattle and Konik horses since 1997 (Figure 6.1). In a comparison of the distribution before the introduction of grazers (period 1982-1986) and a decade after implementing grazing management (period 2000-2010), the population of *H. nummularium* in the Westhoek Nature Reserve has increased by 40 %

(Provoost et al., 2011a). Increasing populations of *H. nummularium* have also been observed after the introduction of large grazers in a comparable coastal dune grassland (Ter Yde, pers. comm. Sam Provoost) and an inland calcareous grassland (Sint-Pietersberg, Lejeune and Verbeke (2009)) as well.

Top: typical grassland vegetation with *Helianthemum nummularium* at the Westhoek Nature Reserve.
Bottom: close-up of flowering *H. nummularium*. ©Bastiaan Notebaert

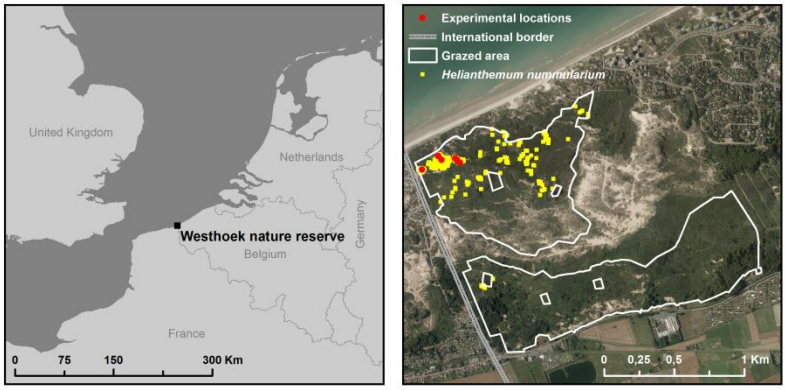

Figure 6.1 - Location of the Westhoek nature reserve where the study took place within Belgium (left) and the extent of the grazing areas, the positioning of the experimental locations and the distribution of *Helianthemum nummularium* in the study area (right). Plant distribution data were adapted from Provoost et al. (2015) and Van Landuyt et al. (2006a). The base layers used in the detailed map are aerial photographs of summer 2012 provided by the Flanders Geographical Information Agency. © AGIV (http://www.agiv.be/)

Exclusion experiment

Three types of exclosures were constructed in grassland containing *H. nummularium* in order to collect evidence for the consumption of *H. nummularium* by different herbivore types and estimate their impact on growth and flowering. The dimensions of the exclosures were 50 x 50 x 50 cm^3 and three different sizes of wire mesh were used to exclude large, large and medium and large, medium and small grazers. As a fourth type, a control plot without wire mesh was marked with poles in order to assess the effects of the complete herbivore assemblage (Table 6.1 and Figure 6.2). The three experimental and control plots were installed at five different locations in the north-western part of the study site (Figure 6.1). In each plot, one visually intact *H. nummularium* plant was selected and marked for future measurements, with a total of 5 measured plants per exclosure type and 20 plants in the whole experiment. The experiment was run during the main growth and flowering period of the species (May 22nd until October

31st, 2013)[3], and weekly maximal plant height was measured and the number of flower buds, flowers, fruits and dry, mature fruits was counted. Grazing events were recorded in case plants missed parts compared with the previous measurements and when the damage was clearly visible. Measurement efforts were lowered to once in every two weeks after the flowering peak from August 9th, 2013 onwards. On the final day of the experimental period, the aboveground parts were harvested and separated between vegetative (stems, branches, and leaves) and reproductive parts (flower buds, flowers, fruits, and mature fruits). Fresh and dry (after drying at 70 °C during 72 h) biomass were subsequently measured for vegetative and reproductive parts separately.

Table 6.1 - The different exclosure types with the mesh size of the fences and the herbivores that were able to enter.

control	large grazer fence	medium grazer fence	small grazer fence
no fence	10.0 cm x 7.5 cm	4.5 cm x 2.0 cm	1.3 cm x 1.3 cm
highland cattle			
Konik horses			
rabbits	rabbits		
hares	hares		
mice	mice	mice	
voles	voles	voles	

[3] According to the Belgian royal meteorological institute (KMI) **May 2013** had an extremely high precipitation (132.5 mm versus 66.5 mm), abnormal low average temperature (11.1 °C versus 13.6 °C) and sunshine duration (136:10 h versus 191:03 h). **June 2013** had a normal precipitation (55.3 mm), average temperature (15.8 °C) and sunshine duration (172:34 h). **July 2013** had an abnormally high sunshine duration (267:43 h versus 200:42 h) and average temperature (20.2 °C versus 18.4 °C), and normal precipitation (65.6 mm). **August 2013** was characterized by normal precipitation (48.3 mm), average temperature (18.6 °C) and sunshine values (213:07 h). **September 2013** had normal precipitation (58.1 mm), average temperature (14.8 °C) and sunshine values (148:27 h). **October 2013** had an abnormally high average temperature (16.3 °C versus 14.7 °C) and normal precipitation (77.5 mm) and sunshine duration (109:50 h) (source: www.meteo.be accessed January 2017).

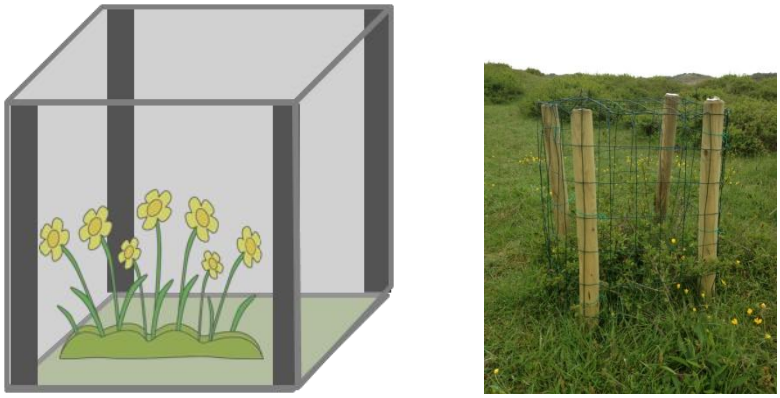

Figure 6.2 - Left: schematic view of an exclosure cage built with 4 wooden poles (black) and encapsulated with wire mesh at the sides and top (grey). Right: picture of an exclosure cage fencing out large grazers.

Endozoochory experiments

In order to quantify dung seed content, samples of horse, cattle and rabbit dung were collected three times in the surroundings of the experimental plots during the fruiting period. In order to avoid cross contamination with seeds present at the soil surface, only superficial dung layers were sampled. For each herbivore, six spatially separated dung samples were taken, dried and cold stratified at 10°C during three weeks. Subsequently, the samples were homogenized and crumbled manually. Subsamples were spread out on a pot containing 1: 1 sterilized sand: organic compost substrate. The pots were put in a germination chamber with a daily light: darkness exposure of 12: 12 hours and daily watering during 23 weeks. The germination of *H. nummularium* was monitored on a weekly basis.

In order to assess the germination success of *H. nummularium* seeds after gut passage, we conducted a feeding experiment using hindgut fermenters (horses) and ruminants (sheep). Three horses and three sheep were selected for the feeding experiment and were kept separately in individual pens which had been cleared from any dung traces in order to prevent contamination. Each animal was fed with a homogeneous mixture of 2500 *H. nummularium* seeds and pellet food. Thereafter, all dung from each individual was collected regularly 6, 12, 24, 48, 72 and 120 hours after

feeding. The fresh mass of each dung sample was measured and dung samples were cold stratified at 10 °C during three weeks. Subsequently, the germinable seed content was quantified similarly as with the dung samples collected at the study site. As a control, 50 untreated seeds were sown in pots without dung.

Epizoochory experiment

The epizoochorous dispersal potential of *H. nummularium* seeds was estimated in an experiment using dummy legs of cattle and horses. We coated PVC tubes (diameter 10 cm, height 100 cm) with genuine animal fur (either cattle fur with relatively long hair length or short haired horse fur) and attached feet of the respective herbivores including original shank fur and hooves at the bottom (Figure 6.3). Seeds were marked with waterproof fluorescent paint (MOTIP Dupli group GmbH) in order to increase their visibility (Lemke et al., 2009). At the start of each experimental run, 13 and 7 seeds were attached to respectively fur and hooves. During each experimental run, the dummy leg was walked over a transect of 300 m with 75 steps/100 m. The remaining seeds were counted after travelling a distance of 100, 200 and 300 m. In order to account for the effects of vegetation structure and weather conditions, the experiment was performed in short grassland and shrub, and in dry and wet weather conditions. In the experimental run simulating wet weather conditions, the fur and seeds were wetted before starting the experiment in rainy conditions, while seeds were attached dry in the experiment with dry weather conditions. Each experimental combination of herbivore type, vegetation type and weather condition was replicated three times.

Figure 6.3 - Left: schematic representation of the dummy leg consisting of a plastic tube coated with animal fur (light grey), shank with original fur (dark grey) and hoof (black). Right: picture of the dummy leg with cattle hoof and fur.

Post-dispersal fate

The post-dispersal establishment success and growth of *H. nummularium* seeds was estimated using different dung treatments, seed densities and plant species combinations. Seeds were sown in cattle and horse dung simulating the deposition site after endozoochory and in a dung-free control treatment. Seeds were sown in three different seed densities using in total 50 (low density), 150 (medium density) and 250 seeds (high density) per planting unit. *Helianthemum* seeds were sown in two-species and four-species combinations with *Agrostis stolonifera*, *Trifolium pratense* and *Trifolium repens* as accompanying species. In the two-species combinations, seeds were either sown in equal (50%-50%) or unequal (20%-80% or 80%-20%) proportions, while only equal proportions were used in the four-species combination (25%-25%-25%-25%). All possible combinations of species mixtures, seed densities, and dung treatments were made. The seed densities used in this experiment were derived from seed densities found germinating in the dung of cattle and horses in a previous study in the same

study area (Cosyns, 2004). Round plastic plant pots (diameter: 15 cm, height: 16 cm) with drainage holes were used as planting units. A 1: 1 mixture of sand and compost was used as a substrate and in the centre of each pot, a plastic ring (diameter: 9 cm; height: 5cm) was inserted and used as the experimental surface in order to reduce the influence of the specific micro-climate at the edges (as in chapter 5, Figure 5.2). Planting pots were put in an unheated greenhouse on felt fabric in order to limit evapotranspiration and to simulate the natural environment. Intact seeds were sown either mixed with cattle or horse dung or on top of the substrate in the control treatment and in one of the predefined seed densities and species combinations. Each combination of dung treatments, seed densities and species mixtures was replicated five times. We used fresh dung that was collected from stabled cattle and horses in order to keep contamination with wild seeds minimal. In the dung samples, rings were filled with 2 cm of homogenized cattle or horse dung and seeds were evenly mixed with the dung layer. In the control treatment, seeds were put directly on the substrate. We estimated contamination with seeds originating from the sand-compost mix, dung or surroundings by using blank pots with either a top layer of seed-free cattle or horse dung or bare substrate. All pots were put in a completely randomized design in order to minimize the impact of the differing micro-climate that might exist in the different parts of the greenhouse. A natural day: night regime was used and plant pots were watered manually on a daily basis. The experiment started on July 27th, 2012 by sowing the seed combinations. The height of the tallest *H. nummularium* plant in each pot was measured after 50, 75, 100 and 300 days by lifting up the hanging parts of the plant. After 300 days, the experiment was stopped and all individuals were counted and harvested by species. All harvested plants were separated into below- and above-ground parts and their dry biomass was measured after oven-drying the plants at 65 °C for 7 days.

Data analysis

In order to quantify the influence of herbivores on the reproductive output of the plants measured in the exclosures, the fructification index (*FI*) and the relative reproductive allocation (*RRA*) were calculated for each individual plant using the formulae as proposed by Dujardin et al. (2011):

$$FI_i\ (\%) = \frac{Fr_i}{FB_i} * 100$$

$$RRA_i\ (\%) = \frac{Wrep_i}{Wveg_i} * 100$$

where Fr_i is the maximal fruit count per individual plant; FB_i is the maximum number of flower buds and $Wrep_i$ and $Wveg_i$ are respectively the biomasses of the reproductive and vegetative parts at the end of the experiment per individual plant.

The absolute germination success (*AGS*) was calculated for each animal in the feeding trial according to Cosyns et al. (2005b):

$$AGS = \left(\sum_{i=t6}^{t120} n_i * W_{Di} * W_{SSi}^{-1}\right) * N^{-1}$$

where n_i is the number of seedlings emerging from the subsample, W_{Di} is the total mass of the dung produced during time interval *i*, W_{SSi} is the mass of the subsample used during the germination trial and *N* is the total number of seeds fed to the animal.

In the epizoochory experiment, seed adhesivity was expressed as the percentage of the seeds remaining on the dummy.

The establishment success (*AS*) of seeds sown in two- or four-species combinations was calculated as follows:

$$AS_i = N_i * D * P_i$$

where N_i is the number of individuals of species *i* counted at the end of the experiment, *D* is the density in which seeds were sown (either 50 (low), 150 (medium) or 250 (high density) seeds) and P_i is the proportion in which

species *i* was sown (either 20%, 50% or 80% in the two-species combinations and 25% in the four-species combinations).

In each census interval, relative growth rates (*RGR*) were derived from the height of the tallest *Helianthemum* plant per pot:

$$RGR = \frac{\ln(H_t) - \ln(H_{t-\Delta t})}{\Delta t}$$

where H_t is the maximum height at time t, $H_{t-\Delta t}$ is the height in the previous interval and Δt is the length of the time interval (Hunt, 1982).

Repeated measures ANOVAs were used to analyse the weekly measurements of height, ramification, flowering and fruiting traits in the exclosure experiment. In case exclosure types differed significantly, Tukey posthoc tests were performed to identify differing cage types. FI, RRA, and biomass data were analysed using one-way ANOVAs with exclosure type as the independent factor. RRA was arcsine square root transformed in order to meet the assumptions for parametric testing. Absolute germination success was analysed using Kruskal-Wallis tests. Seed adhesivity was analysed after an arcsine square root transformation using a repeated measures ANOVA with herbivore species, body parts, vegetation types, weather conditions and walking distance as independent variables with herbivore species: body part and vegetation type: weather condition as interactive terms. Establishment success and biomass were respectively arcsine square root transformed and analyzed using ANOVAs with dung type, seed density, the proportion of *H. nummularium* and accompanying species as independent factors and dung type: seed density and proportion: accompanying species as interaction terms. The relative growth rate of *H. nummularium* (*log(RGR+1)* transformation) was analyzed using a repeated measures ANOVA with dung and seed density as independent factors and a nested proportion: accompanying species term. Standardized major axis regressions (SMA) were used to evaluate the bivariate relationships between root and shoot biomass with dung type, seed density and accompanying species as fixed factors (Warton et al., 2006). Using the method of Warton et al. (2012), we first tested the data for common slopes between dung types, seed densities or accompanying species. In case no differences in slopes were found, we tested if elevations (or *y*-intercepts)

differed between the factors as the fitted lines may both represent a shift along their common slope and/or a shift in elevation.

All analyses were performed using R version 3.3.1. (R Core Team, 2016b). The 'smatr' version 3.4-3 (Warton et al., 2012) was used for standardized major axis regressions.

Results

Endozoochorous dispersal potential

Visual evidence of grazing incidences were recorded three times during the exclusion experiment as two *Helianthemum nummularium* plants got grazed in the uncaged plots and one individual in the plots excluding large grazers. No visible signs of grazing were found in the other cage types. Grazer fences did not affect flowering and fruiting, although at the end of the experiment plants were higher and more ramified plants in the plots with medium-mesh fences (Table 6.2 and Figure 6.4). At the end of the growing season, fructification indices, relative reproductive allocation, and biomass were not affected by the different cage types (Table 6.3).

After 23 weeks, no *H. nummularium* seedlings had emerged from the cattle, horse and rabbit dung collected at the study site. Although seeds were able to germinate in the feeding experiment, the absolute germination success of ingested seeds (horses: 0.105 ± 0.105 %; sheep: 0.040 ± 0.023 %) was significantly lower compared to intact seeds on a bare substrate (23.667 ± 4.631 %, p= 0.015).

Table 6.2 - Results of the repeated measures ANOVA with cage and time as independent factors and respectively height, ramification and flower bud, flower, fruit and mature fruit counts as dependent variables.

measured variable	factor	*df*	*F* value	*p*
height	cage	3	0.922	0.453
	time	1	60.4	<0.001
	cage x time	3	4.09	0.007
ramification	cage	3	0.568	0.644
	time	1	250.38	<0.001
	cage x time	3	12.78	<0.001
flower buds	cage	3	0.359	0.783
	time	1	152.233	<0.001
	cage x time	3	1.262	0.287
flowers	cage	3	0.354	0.787
	time	1	28.053	<0.001
	cage x time	3	0.553	0.646
fruits	cage	3	0.104	0.956
	time	1	9.025	0.003
	cage x time	3	0.211	0.889
mature fruits	cage	3	0.149	0.929
	time	1	52.121	<0.001
	cage x time	3	0.169	0.917

Table 6.3 - Average values and standard errors for fructification index, relative reproductive allocation and biomass by exclosure type and p-values resulting from the ANOVA analysis.

variable	control	large	medium	small	*p*
FI (%)	80.42 ± 5.90	88.52 ± 2.02	82.01 ± 4.59	84.14 ± 5.19	0.644
RRA (%)	2.97 ± 2.53	3.36 ± 3.06	1.69 ± 1.02	4.95 ± 4.43	0.989
biomass (g)	0.67 ± 0.21	0.77 ± 0.53	2.14 ± 0.87	0.71 ± 0.27	0.197

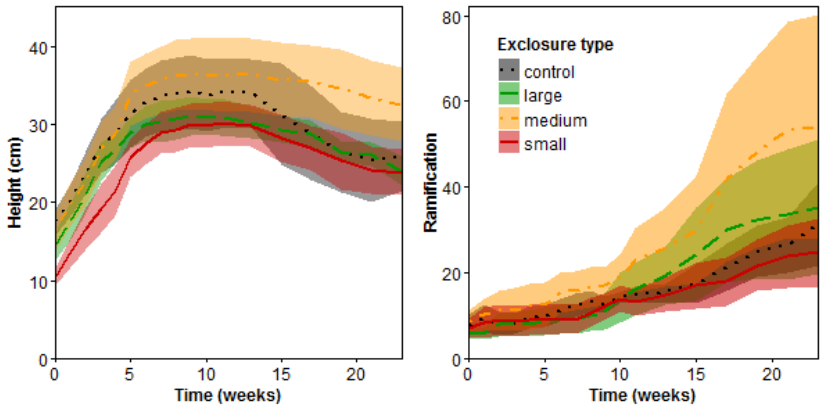

Figure 6.4 - Evolution of average plant height and ramification by exclosure types. Mean values and standard errors are plotted.

Epizoochorous dispersal potential

The fur and hooves of cattle and horses were able to transport seeds in the epizoochory experiment although the distance seeds travelled greatly differed between herbivore species, body parts, vegetation types and weather conditions (Table 6.4). High seed numbers were able to travel long distances attached to the skin of cattle and horses when the vegetation was short, while seeds almost immediately detached from the skin in higher vegetation (Figure 6.5). Furthermore, the weather conditions greatly affected seed adhesivity in short vegetation as seeds remained attached to the skin significantly longer under rainy conditions. Although seeds detached more easily from the hooves of cattle and horses, the effects of weather conditions and surrounding vegetation were less relevant as hooves transported more seeds through high vegetation compared with skin (Figure 6.5).

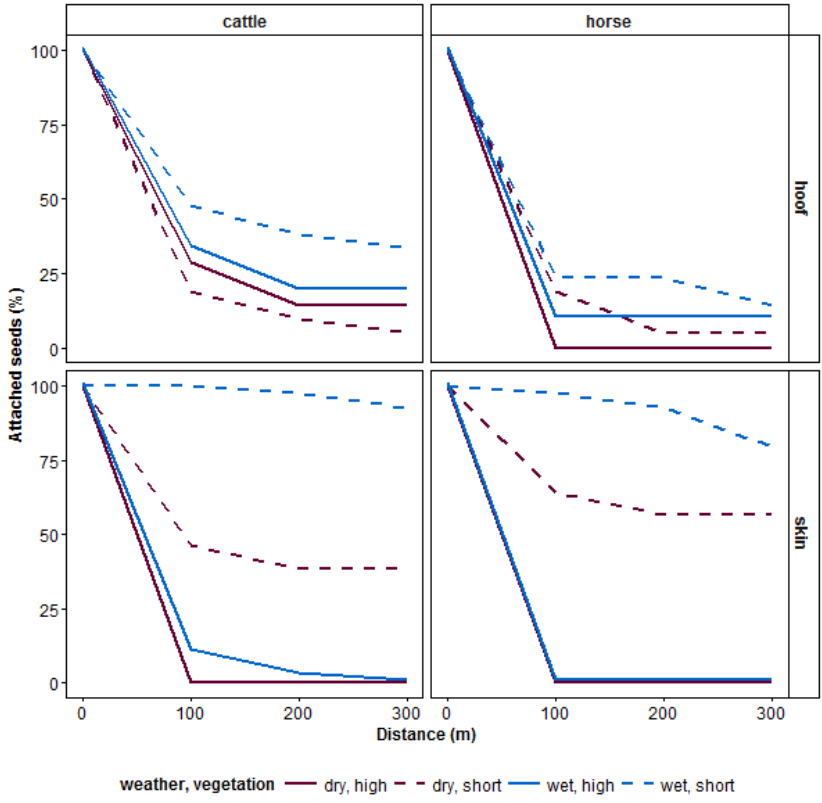

Figure 6.5 - Seed adhesivity during the epizoochory experiment by herbivore type, body part, vegetation and weather conditions. See Table 6.4 for full ANOVA results.

Table 6.4 - Results of the ANOVA analysis of seed adhesivity during the epizoochory experiment using herbivore species, body part, vegetation and distance as independent factors.

factor	***df***	***F* value**	***p***
herbivore	1	6.294	0.021
body part	1	8.209	0.005
Herbivore x body part	1	2.152	0.144
vegetation	1	100.808	<0.001
weather	1	21.334	<0.001
vegetation x weather	1	7.415	0.014
distance	1	166.563	<0.001

Post-dispersal fate

Helianthemum nummularium grew significantly faster when sown in cattle dung compared to the control treatment (p<0.001) and horse dung (p=0.002). Furthermore, growth was enhanced by horse dung compared to bare soil (p=0.007) while seed densities and species combinations did not alter the growth rate of plants (Table 6.5 and Figure 6.6).

Table 6.5 - Repeated measures ANOVA results evaluating the effect of dung, differing seed densities, accompanying species and seed proportions on the relative growth rate (RGR) of *Helianthemum nummularium* plants over time.

factor	*df*	*F* value	*p*
dung	2	23.322	<0.001
seed density	2	0.383	0.682
time	1	83.096	<0.001
accompanying species	3	1.171	0.321
H. nummularium proportion	6	0.830	0.547
dung x seed density	4	0.892	0.469
dung x time	2	1.901	0.151
seed density x time	2	0.025	0.975

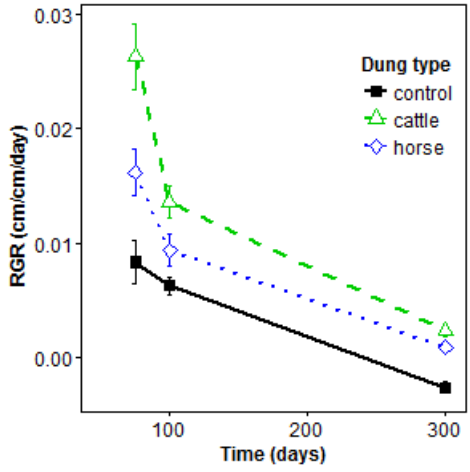

Figure 6.6 - Average relative growth rate (RGR) of *Helianthemum nummularium* plants by dung type during the establishment experiment

Dung did not affect the establishment success and biomass of *H. nummularium* plants. More plants established when seeds were sown in low densities, but the biomass of individual plants did not differ among seed densities. Also, more *H. nummularium* plants established and reached a higher individual biomass when sown in combination with *Agrostis stolonifera* compared to the mixtures containing *Trifolium pratense* or *Trifolium repens* (Figure 6.7 and Table 6.6). The root: shoot ratio differed significantly between dung types, seed densities and species combinations (Figure 6.8). The shoot biomass was relatively higher when plants were growing in horse dung compared to the control and cattle dung treatments (p<0.001). Furthermore, *Helianthemum* plants invested more in shoot biomass when the seed density was low (p<0.001). Lower root: shoot ratios were found in the combinations containing the four tested species (p<0.001) and *T. pratense* (p= 0.0311).

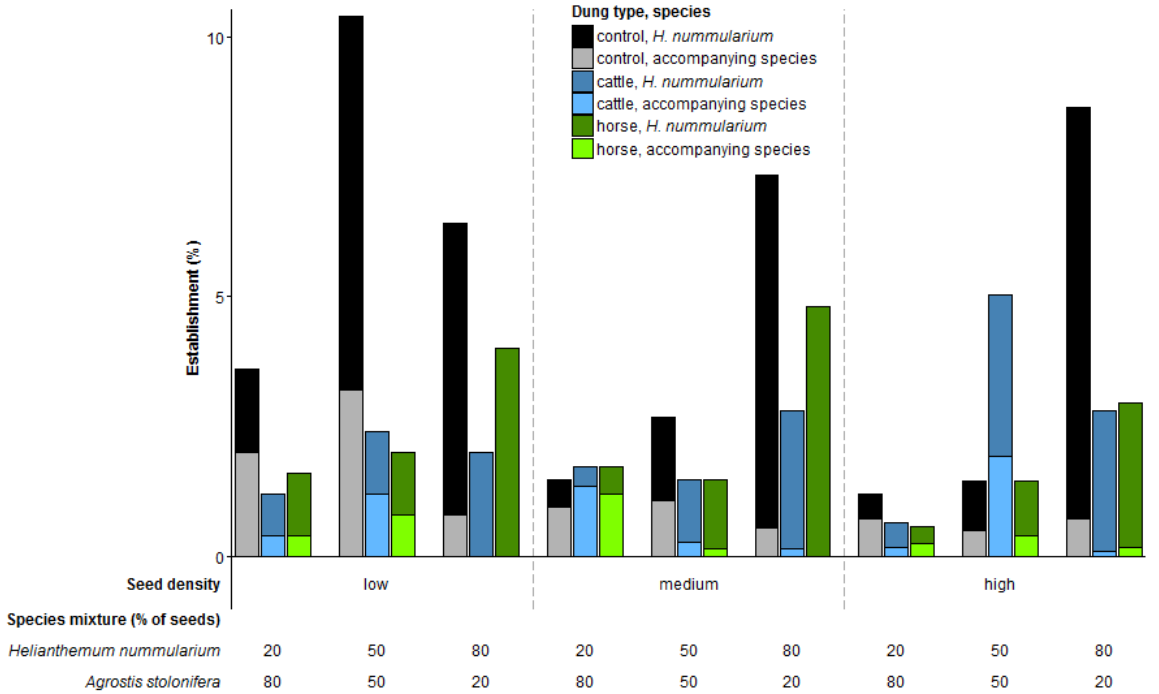

Figure 6.7a - Establishment success of *H. nummularium* growing in two-species combinations with *Agrostis stolonifera* in different seed densities, species mixtures, and dung types.

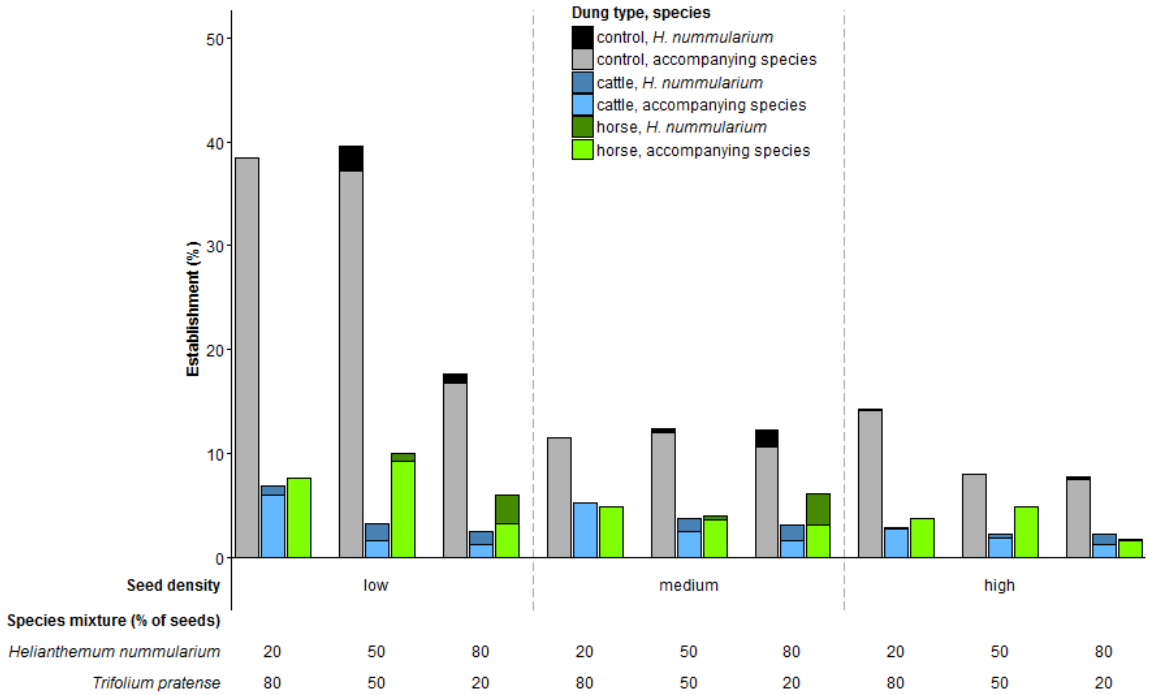

Figure 6.7b - Establishment success of *H. nummularium* growing in two-species combinations with *Trifolium pratense* in different seed densities, species mixtures, and dung types.

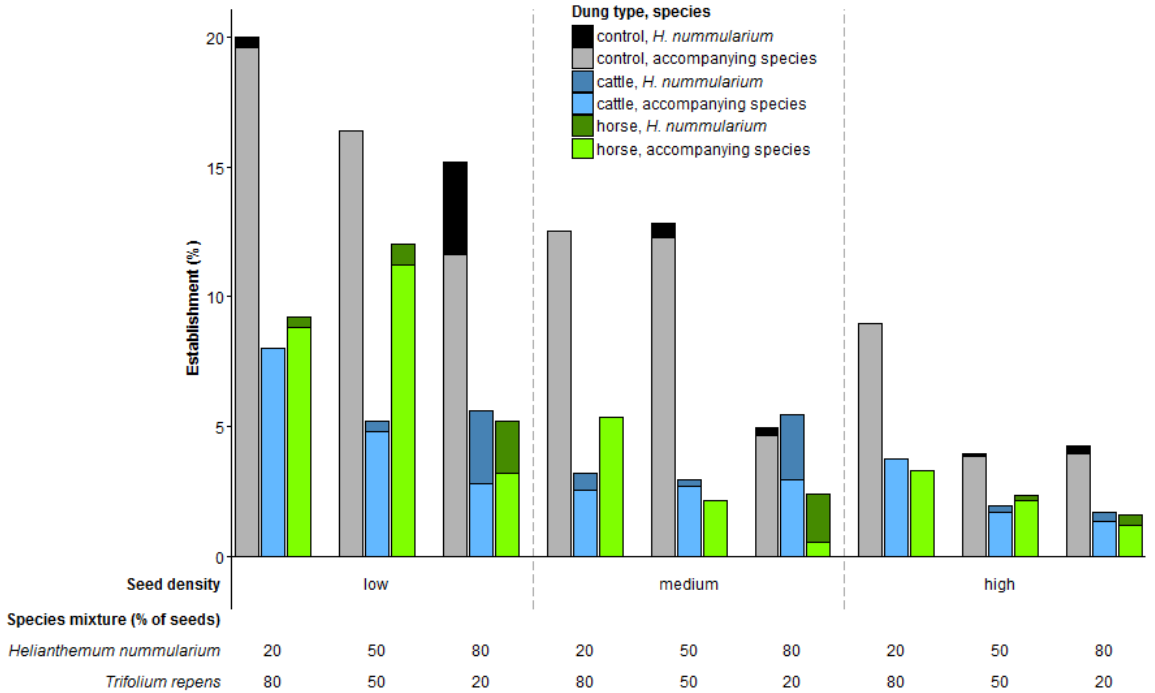

Figure 6.7c - Establishment success of *H. nummularium* growing in two-species combinations with *Trifolium repens* in different seed densities, species mixtures, and dung types.

Table 6.6 - Results of the ANOVA analysis of the establishment success and individual biomass of *Helianthemum nummularium* with dung types, seed densities, the proportion of *H. nummularium* and accompanying species as independent factors.

measured variable	**factor**	***df***	***F* value**	***p***
establishment success	dung	2	0.340	0.712
	seed density	2	4.642	0.010
	H. nummularium proportion	1	24.965	<0.001
	accompanying species	2	38.798	<0.001
	dung: seed density	4	0.684	0.603
	H. nummularium proportion x accompanying species	2	0.242	0.785
biomass	dung	2	1.319	0.271
	seed density	2	1.028	0.361
	H. nummularium proportion	1	1.846	0.177
	accompanying species	2	4.495	0.013
	dung: seed density	4	0.305	0.874
	H. nummularium proportion x accompanying species	2	0.703	0.497

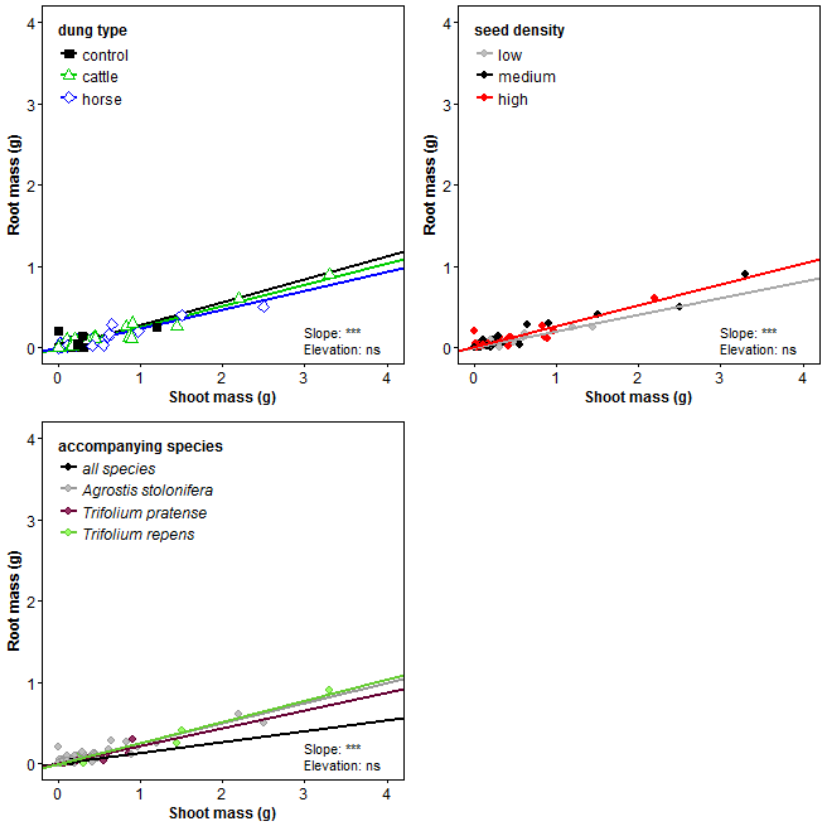

Figure 6.8 - Bivariate plots of standardized major axis regressions (SMA) between root and shoot biomass of *Helianthemum nummularium* plants across dung treatments (cattle, horse or no dung), seed densities (low, medium or high) and in different species combinations (in either two-species combinations with *Agrostis stolonifera*, *Trifolium pratense* or *Trifolium repens*, or in a four-species combination with all before mentioned species). 'Slope' indicates differences in SMA slopes and 'Elevation' indicates differences in SMA elevations (i.e. *y*-axis intercept). Significant differences in slopes and elevations are marked with asterisks with ***: $p<0.001$, **: $0.001<p<0.010$, *: $0.01<p<0.050$ and ns: $p>0.050$.

Discussion

Although consumption of *Helianthemum nummularium* by large herbivores (cattle and/or horses) and by medium-sized herbivores (rabbits and/or hares) was occasionally observed in the weekly measurement of grazing damage, grazing only slightly impacted the growth phase while the reproductive phase was not affected. The high fructification index (FI) found in our research (80.4 to 88.5 %) suggests that *H. nummularium* grows in an optimal environment with sufficient light and low competition levels. Dujardin et al. (2011) found comparable FI in a short grassland environment (86.3 %) while FI lowered when *H. nummularium* individuals were growing in tall or shrub-encroached grassland to respectively 56.1 and 49.1 %. They concluded that *H. nummularium* shifts the energy allocation from reproductive towards vegetative growth in competitive situations. The high investment in reproductive organs found in our research might also result in an increased probability of seed dispersal events.

Although grazing observations were rare, the magnitude of the damage caused by grazing differed among herbivore sizes. During the course of our experiment, medium-sized grazers only removed some branches and inflorescences while large herbivores consumed almost the whole plant. If this trend continues, the greater consumption by large herbivores might increase the probability of seed ingestion, and hence endozoochorous dispersal. On the other hand, successful endozoochorous dispersal was not confirmed in the dung samples taken from cattle, horses, and rabbits. Although dung was sampled three times during the fruiting season and many seedlings of a variety of plants appeared during the six-month incubation period, no *H. nummularium* seedlings could be identified. Earlier, Cosyns et al. (2005a) found very low numbers of *H. nummularium* seeds germinating from cattle and horse dung sampled in the Westhoek nature reserve (respectively 0.6 % and < 0.1 % of the total number of emerging seedlings) which was also confirmed by the findings of Dai (2000) in an alvar limestone grassland in Sweden. In the extensive study of the diet of introduced ungulates in coastal areas, Lamoot et al. (2005a) documented only one incidence of the consumption of *H. nummularium* by donkeys in a nearby coastal area. The fact that the species has a very low cover in the study area (0.97 % according to Cosyns et al. (2005a)) and its rather low forage value according to the BiolFlor database (Kühn et al., 2004) and the

presence of more nutritive grass species growing in association with *H. nummularium* lowers the probability of accidental and targeted grazing behaviour. Moreover, the amount of sampled dung in the present study might not be sufficient to detect *H. nummularium* seeds, although the amount of dung collected during one sampling period equalled the 2.5 l of dung collected by Cosyns et al. (2005a), with an exception for rabbit dung. Furthermore, we retrieved very low numbers of germinable seeds from sheep and horse dung after feeding a known number of seeds (respectively 0.040 % and 0.105 %). The absolute germination success of ingested seeds was lower than in a previous study by Cosyns et al. (2005b) while the germinability of uningested seeds on bare soil, and hence the quality of the seed batch, was comparable with the results of Cosyns et al. (2005b). In contrast, D'hondt and Hoffmann (2011) recorded a much higher survival rate of ingested *H. nummularium* seeds compared to intact seeds in a similar feeding experiment using cattle. Although neither we nor Cosyns et al. (2005b) fed seeds to cattle, the survival rate of *H. nummularium* seeds was substantially lower in horses, sheep, donkeys and rabbits (Cosyns et al., 2005b) than with D'hondt and Hoffmann (2011). This suggests that the few seeds that remain viable after passing the intestinal tract have a high germination probability. Seeds of Cistaceae species typically have a high incidence of physical seed dormancy caused by the presence of a hard and water-impermeable seed coat and, therefore, do not germinate easily (Baskin and Baskin, 2001; Thanos et al., 1992). Mechanical scarification and thermal pre-treatment (e.g., fire) of the seeds are known practices to induce germination, but even then germination rates often remain low (Pérez-García and González-Benito, 2006). Cistaceae species have often been linked to endozoochorous dispersal as their hard seed coats might protect the seeds from being digested in herbivore guts and the several processes following ingestion may result in enhanced germination (Jaganathan et al., 2016; Ramos et al., 2006). On the other hand, intense chewing might completely crush seeds. In a simulated digestion experiment, Milotić and Hoffmann (2016b) found an increased germination of *H. nummularium* seeds after mechanical scarification with sandpaper while slower and less frequent germination was noted after chewing with cattle teeth, the chemical environment of hydrochloric acid and pepsin in the stomach and the raised temperature during the passage through the herbivore's guts. The reduced germination success of gut-passed seeds found in many studies (e.g., Cosyns et al. (2005b), Dai (2000), Pakeman and Small (2009)) suggests

a high cost of this mode of dispersal. Furthermore, the different digestive systems found in herbivores (ruminants (e.g., cattle and sheep) and hindgut fermenters with (e.g., rabbits and hares) or without re-ingestion of cecotropes (e.g., horses and donkeys)) might result in different survival rates of the ingested seeds (Will and Tackenberg, 2008). Cosyns et al. (2005b) indeed found a higher germination success when *H. nummularium* seeds were ingested by sheep compared to horses and rabbits.

Previous studies already illustrated the role of cattle, horses and rabbits in the external transport of seeds (e.g., Couvreur et al. (2004b), Couvreur et al. (2005a), Couvreur et al. (2005b), Fischer et al. (1996), Mouissie et al. (2005a)), and our results clearly support the potential role of epizoochory in the dispersal of *Helianthemum* seeds. In our experiment, cattle fur and hooves were able to transport seeds over a longer distance than horses. Furthermore, Couvreur et al. (2005b) concluded that cattle dispersed more seeds than horses suggesting that cattle are better seed dispersers. Fur type, length, and growth angle are decisive factors in determining seed retention times (Couvreur et al., 2004b; Couvreur et al., 2005b; Fischer et al., 1996). The fur of the hardy cattle breeds that are commonly used in nature management (such as Scottish Highland cattle and Galloway cattle) generally has longer and rather undulated hairs which are implanted at a larger angle. Therefore, it has a higher capacity to attach seeds compared to horse fur with shorter, straight hairs implanted at small angles (Couvreur et al., 2005b). Our results also suggest that the relevance of hooves in seed dispersal should not be neglected. Although the adhesivity is lower compared to fur, the probability that seeds of short plant species such as *H. nummularium* get attached to hooves could be higher compared to fur (Schulze et al., 2014). Furthermore, seeds remained longer on cattle hooves. This could be due to the larger structural diversity found in cattle hooves with two dew-claws and two separate soles. Weather conditions greatly affected the epizoochorous dispersal capacity of *Helianthemum* seeds as seeds remained attached substantially longer in wet conditions. The seeds of *H. nummularium* have a rather smooth surface and lack any obvious adaptations to epizoochorous dispersal. Seeds of certain species in the *Helianthemum* genus become covered with a layer of mucilage as soon as they come in contact with a wet substrate (Baskin and Baskin, 2001). This sticky layer also develops at this surface of *H. nummularium* seeds and causes the better adhesivity in wet conditions. Furthermore, seeds became

detached almost immediately in high vegetation. Although seeds remained slightly longer on cattle and its hooves, the spatial configuration of the terrain is decisive in determining seed dispersal distances. The fact that the Westhoek reserve is a spatially heterogeneous landscape with alternating grassland of diverse height and shrub vegetation (Ebrahimi, 2007) could, therefore, constrain the epizoochorous dispersal of *Helianthemum* seeds.

Besides animal traits and environmental conditions, plant attributes such as diaspore release height, plant abundance, and seed morphology may also affect seed adhesivity (Albert et al., 2015b). Seeds released at a low height are less likely to get attached to animal fur, unless the animal is lying down or grazing although attachment to the head while grazing is rather unlikely (Fischer et al., 1996; Mouissie et al., 2005a). Despite the fact that seeds of *H. nummularium* are able to disperse through both endozoochory and epizoochory, the probability of being ingested or attached is probably low since this species has a low coverage and low numbers of cattle and horses are present in the study area. On the other hand, only a few seeds need to be transported to a suitable habitat to establish a new population.

In order to quantify the impact of each dispersal method on plant communities, we should take into account the post-dispersal fate of seeds. The environment of the deposition sites largely differs between the zoochorous dispersal methods. In the case of endozoochory, large amounts of seeds are concentrated in dung pat islands, while seeds are dropped in a much more scattered way in case of epizoochory. In our experiment, dung addition clearly enhanced the growth rate of *H. nummularium* seedlings compared to the dung-free control treatment, but the establishment success and plant biomass were not affected. In contrast, a germination trial in which low densities of *H. nummularium* seeds were sown in cattle and horse dung resulted in a lower germination success and longer germination times in case seeds were sown in dung (Milotić and Hoffmann, 2016c). The increased growth rate of *H. nummularium* seedlings in cattle dung found in this study is also confirmed by earlier research where *H. nummularium* was grown in a completely competition-free environment (1 plant per pot design, Milotić and Hoffmann (2016a)). Furthermore, in our experiment seedling establishment success was mostly affected by seed density and combination with other species while biomass was only affected by the accompanying species. The accompanying species were chosen according to their differences in morphology and ecological strategy with *Agrostis*

stolonifera as an indifferent (*CSR*) species and creeping grass, *Trifolium pratense* as a competitive species (*C*) and tall forb, and *Trifolium repens* as an indifferent (*CSR*) and creeping forb species while *H. nummularium* is classified as a competitive and stress tolerant (*CS*) dwarf shrub (Grime, 1977). Our results suggest that *T. pratense* is a competitively superior species to *H. nummularium* as the establishment success and biomass of the latter species significantly decreases when grown in combination with *T. pratense*. Furthermore, the lowered root: shoot ratio in the combinations including *T. pratense* (both in two-species as four-species combinations) suggests that *H. nummularium* allocates more biomass in aboveground plant parts due to competition for aboveground resources (e.g., light and CO_2) (Brouwer, 1962; Poorter and Nagel, 2000). As dung often contains high numbers of endozoochorously dispersed seeds belonging to a wide array of species (Cosyns et al., 2005a), dung pat islands can be regarded as a medium with high levels of inter- and intraspecific competition. Therefore, we can assume that the post-dispersal fate of *H. nummularium*, as a rather slow-growing perennial, largely depends on the ecological strategy and seed densities of the other species present in the dung pat (endozoochory) or the plant composition at the deposition site (epizoochory). As trampling can create bare soil and *H. nummularium* germinates best in a dung-free environment without competition, we could argue that hoof-epizoochory, in particular, could explain some of the observed patterns. Another strategy would be to delay germination until the initially high competition levels following seed deposition are lowered, although germination should take place within the first year after deposition as *H. nummularium* generally has transient seeds (Thompson et al., 1997).

Other non-dispersal related factors might contribute to the increasing appearance of *H. nummularium* as well. The species is known from open chalk grassland-scrub mosaics. Extensive grazing is expected to create and/or maintain such mosaic structure (e.g., Ebrahimi et al. (2007)) and might have led to the expansion of the species since the introduction of large herbivores in the area. The beneficial effects of extensive grazing on the biodiversity and structural diversity of dune grasslands are widely recognized (e.g., Kooijman and de Haan (1995), ten Harkel and van der Meulen (1996)). According to Proctor (1956), *H. nummularium* resists moderate levels of trampling and grazing but hardly persists in thick ungrazed turf. On the other hand, Pittarello et al. (2016) found a decrease of

H. nummularium after installing temporary night camp areas and mineral mix supplements that attract cattle and locally increase trampling and grazing. Therefore, the extensive grazing management in our study area might be just perfect to reduce the cover with perennial grasses and to create an ideal environment for the establishment of *H. nummularium*.

Conclusion

We conclude that the increased presence and coverage of *H. nummularium* after the introduction of large domestic herbivores is the result of the interplay between various ecological processes. Herbivores can play a role in the dispersal of *H. nummularium* seeds via internal (endozoochory) and external ways (epizoochory) while their selective grazing behaviour with diet preference for grasses most probably creates an appropriate sufficiently open mosaic grassland-scrub environment for plant establishment and maintenance. Although very few seeds pass the digestive system alive, the presence of dung does not seem to affect the establishment success of *Helianthemum* plants and even enhances the growth rate. Hence, endozoochory might be playing a role in the dispersal of this species. Furthermore, epizoochorous dispersal, especially by hooves, should also be considered as one of the plausible dispersal mechanisms.

Acknowledgments

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PART II: THE FATE OF DUNG

The role of dung beetles in dung removal and secondary seed dispersal

To the ancient Egyptians, the ball-rolling dung beetle *Scarabaeus sacer* was a symbol of the sun god (KHM, Vienna, Austria) ©Bastiaan Notebaert

7 Linking functional group richness and ecosystem functions of dung beetles: an experimental quantification

Tanja Milotić, Stijn Quidé, Thomas Van Loo and Maurice Hoffmann

Experimental plots at Kalmthout, Belgium (August 2014).

Modified from Milotić, T., Quidé, S., Van Loo, T., Hoffmann, M. (2017) Linking functional group richness and ecosystem functions of dung beetles - an experimental quantification. Oecologia, 183(1), 177-190, DOI: 10.1007/s00442-016-3756-5

Abstract

Dung beetles form an insect group that fulfils important functions in terrestrial ecosystems throughout the world. These include nutrient cycling through dung removal, soil bioturbation, plant growth, secondary seed dispersal and parasite control. We conducted field experiments at two sites in the northern hemisphere temperate region in which dung removal and secondary seed dispersal were assessed. Dung beetles were classified in three functional groups, depending on their size and dung manipulation method: dwellers, large and small tunnelers. Other soil inhabiting fauna were included as a fourth functional group. Dung removal and seed dispersal by each individual functional group and combinations thereof were estimated in exclusion experiments using different dung types. Dwellers were the most diverse and abundant group, but tunnelers were dominant in terms of biomass. All dung beetle functional groups had a clear preference for fresh dung. The ecosystem functions of dung removal and secondary seed dispersal provided by dung beetles were significant and differed between functional groups. Although in absolute numbers more dwellers were found, large tunnelers were disproportionally important for dung burial and seed removal. In the absence of dung beetles, other soil inhabiting fauna, such as earthworms, partly took over the dung decomposing role of dung beetles while most dung was processed when all native functional groups were present. Our results, therefore, emphasize the need to conserve functionally complete dung ecosystems in order to maintain full ecosystem functioning.

Keywords: dung beetles, dung removal, functional groups, secondary seed dispersal

Introduction

In recent decades, there has been an increasing interest in ecosystem functions and services and their value for human economy and well-being (Millennium Ecosystem Assessment, 2005; Naeem et al., 2002). The signs of a new human induced species extinction wave (Barnosky et al., 2011) resulted in a multitude of studies of the relation between biodiversity and ecosystem functioning (Cardinale et al., 2002; Reiss et al., 2009) in an attempt to model the future effects of performance of incomplete ecosystems and to find solutions to biodiversity loss. Many of these studies have shown the importance of preserving biodiversity to maintain ecosystem functioning and the resulting services (e.g., Schwartz et al. (2000), Srivastava and Vellend (2005), Balvanera et al. (2006)). Often, mixtures of species outperform the best functioning monoculture for a particular function through facilitation or niche complementarity (so-called 'transgressive overyielding') (Cardinale et al., 2002; Schmid et al., 2008). However, an often posed criticism on biodiversity and ecosystem functioning research is that many of these studies used artificially composed and restricted sets of species and were performed under strictly controlled abiotic conditions (Finn, 2001; Naeem and Wright, 2003; Slade et al., 2007). Field studies in which specific species or groups of species are experimentally included or excluded in the measurement of ecosystem functions can overcome this problem. The use of existing species assemblages without manipulating the environmental conditions is therefore recommended in order to study specific ecosystem functions. Dung beetles are an example of suitable model organisms in ecosystem functioning research as they are globally distributed and abundantly present (Hanski and Cambefort, 1991b). Most of the ecosystem functions provided by dung beetles are linked to the removal and underground burial of dung (Losey and Vaughan, 2006; Nichols et al., 2008), which results in bioturbation increase and enhanced nutrient cycling. While digging their nests, dung beetles perturb the soil, aerate it, change the hydrological properties (Brown et al., 2010) and altogether alter the structure of the soil top layers (Bang et al., 2005a). In Western Europe for instance, the presence of the tunnelling species *Typhaeus typhoeus* on its own is estimated to move 450 kg of soil per hectare annually (Brussaard and Visser, 1987).

Dung beetles play an important role in nutrient cycling as they remove and bury vertebrate faeces containing considerable amounts of nutrients (Hanski, 1987), which increases the availability of these nutrients in the ecosystem (Yamada et al., 2007), for plant growth (Borghesio et al., 1999) and crop yield (Miranda et al., 2000) and reduces the emission of the greenhouse gas methane (Penttilä et al., 2013). An indirect effect of dung beetle activity is their role as pest control agents. Dung plays an important role in the lifecycle of many parasites infecting vertebrates, such as flatworms and nematodes, but also attracts external pest species such as flies. By reducing the aboveground amount of dung, dung beetles have a negative impact on the reproduction success of these pest species (Bryan, 1973) and reduce the infection rate of grazing vertebrates (Fincher, 1973; Fincher, 1975). Overall, a great economic value has been attributed to dung beetles. Losey and Vaughan (2006) estimate the annual economic value of dung beetles in the United States at 380 million dollars, remarking that this number is an underestimate.

Next to these ecosystem functions with direct economic benefits, dung beetles play an important role in plant ecology through the process of secondary seed dispersal (Andresen, 2002b; Feer, 1999). Endozoochory, or dispersal of propagules by herbivores after gut passage, is a commonly found dispersal mechanism and enables long-distance dispersal of plant seeds (Nathan et al., 2008). Dung beetles can act as secondary dispersers of the seeds already present in dung and reshape plant communities (Andresen, 2003; Nichols et al., 2008). Secondary seed dispersal by dung beetles may result in a higher seedling establishment by reducing the risks of predation and mortality, directing dispersal to more favourable locations for germination and decreasing scramble competition for space and nutrients by seedlings (Andresen, 1999; Andresen and Feer, 2005; Andresen and Levey, 2004; Nichols et al., 2008). Given their ecological and economic importance, it is essential to understand the implications of a changing community composition for the ecosystem functions dung beetles fulfil, also because they are sensitive to habitat modification (Nichols et al., 2008), which is a global problem. In comparison with tropical biomes, few studies have been published on dung beetle ecology in temperate regions, and the knowledge of their role in ecosystem functions and services in Western Europe is rather fragmentary. Nevertheless, as dung beetles are abundantly present beyond the tropics, they are very well suited for biodiversity and

ecosystem functioning research in many ecosystems. Furthermore, it is easy to measure rates of dung and seed removal and dung pats which are spatially and temporally separated patches can simply be replicated and manipulated (Finn, 2001).

As a division into functional groups based on body size and method of dung exploitation can be made, the effects of excluding certain groups can be assessed experimentally. One common way of classifying dung beetles is based on functional traits according to the beetles' nesting behaviour (Doube, 1990). Telocoprids (rollers) make dung balls and roll it away from the dung pile before burying it, while paracoprids (tunnelers) bury dung below the dung pile and endocoprids (dwellers) do not significantly relocate dung (Hanski and Cambefort, 1991b). The use of functional groups is relevant as changes in certain ecosystem functions can be greater when an entire functional group is lost compared to the loss of an equivalent number of species spread over all functional groups (Larsen et al., 2005; Slade et al., 2007). Since the amount of dung that is removed strongly correlates to the mean female body size (Horgan, 2001) and larger beetle species tend to be more extinction prone and functionally efficient (Larsen et al., 2005) an extra division based on body size is appropriate.

In this study, we aim to link dung beetle assemblages with dung removal and secondary seed dispersal in the temperate Atlantic biogeographical region. As we expect that dung removal and secondary seed removal are closely linked to nesting behaviour and beetle size, we designed a field experiment in which different combinations of functional groups were granted or denied access to experimental dung piles. We also hypothesize that dung beetle assemblages, and therefore the ecosystem functions of dung beetles, vary between sites and seasons and that dung originating from different herbivore species might attract different sets of dung beetle species. Therefore, species diversity and abundance were recorded during the experiments and the study was simultaneously performed in two locations using different dung types and was replicated in two seasons.

Materials and methods

Site description

The study took place in two nature reserves situated within the Atlantic biogeographical region in Belgium: the 'Zwindunes and Zwinpolders' and 'De Zoom-Kalmthoutse heide'. The 'Zwindunes and Zwinpolders' (further referred to as 'The Zwin') are situated in the coastal dune ecoregion at the most north-eastern section of the Flemish coast between the centre of Knokke-Heist and the Dutch-Belgian border. 'De Zoom-Kalmthoutse heide' (further referred to as 'Kalmthout') is located in the Campine ecoregion and is a cross-border park on the Dutch-Belgian border near the centre of Kalmthout (Appendix, Figure A 7.1) (Couvreur et al., 2004c). Free-ranging semi-wild grazers were introduced in The Zwin area in 2002 and in Kalmthout in 1997. During this study, the grazed areas in The Zwin and Kalmthout respectively had a surface area of 98 ha and 639 ha. Grazing pressure and animal species and breeds differ between sites with Highland cattle (*Bos taurus*, 0.52 animal units (AU)/ha), Konik horses (*Equus ferus caballus*, 0.15 AU/ha) and Flemish sheep (*Ovis aries*, 0.20 AU/ha) in The Zwin area and Galloway cattle (*Bos taurus*, 0.20 AU/ha) in Kalmthout. Furthermore, a herded flock of Drenthe sheep (*Ovis aries*, 0.20 AU/ha) is present in Kalmthout.

In each study site an experimental area of approximately 300m² was fenced out in order to avoid disturbance by grazers during the field experiment, but allowing access of dung beetles associated with the large herbivores in the area. The exclosure in The Zwin was located in the subarea 'Kleyne Vlakte' (51°21'21"N, 3°20'41"E, elevation 3 m a.s.l.) which is a relatively open landscape consisting mainly of grassland (most affinity with *Arrhenatherion* grassland communities), while the exclosure in Kalmthout was located in the subarea 'Biezenkuilen' (51°24'54"N, 4°25'10"E, elevation 18 m a.s.l.) and is covered by heathland (*Genisto anglicae-Callunetum typicum*). The soil in both areas consists predominantly of sand: 85.7% sand, 7.6% silt and 6.7% clay (USDA texture class loamy sand) in The Zwin and 97.4% sand, 1.5% silt and 1.1% clay (USDA texture class sand) in Kalmthout.

Functional groups

We focussed on quantifying two ecosystem functions implemented by different functional groups of coprophagous fauna: dung removal and the redistribution of endozoochorously dispersed seeds (secondary seed dispersal). Dung beetles were classified in three functional groups according to their dung processing behaviour: rollers, tunnelers, and dwellers. A further classification was made based on body size. Tunnelers and rollers were classified as either small or large depending on their ability to move through a 1 cm^2 mesh (square mesh with side lengths of 1 cm) (Slade et al., 2007). Dwellers are generally small beetles and as they do not actively move dung, no further size differentiation was made. Furthermore, other soil fauna, such as earthworms, are often major dung decomposers in Northern Europe (Gittings and Giller, 1999; Gittings et al., 1994). Therefore, the dung removing behaviour of all other soil fauna able to go through 1 cm^2 mesh but stopped by 1 mm^2 mesh was measured as well (Appendix, Figure A 7.2).

Experimental design

The experiment was replicated using three dung types of domestic grazers present in or close-by the study sites: cattle, horses, and sheep. Three sections were marked within each fenced exclosure, one for each dung type. In each section, the experimental units and sampling units were put in a fully randomized design (Appendix, Figure A 7.3). Individual experimental units and sampling units were on a spacing of 60 cm, and different sections were at a distance of 2 m.

Eleven types of experimental units were designed by combining different kinds of ground screens, vertical 'walls' and/or 'ceilings' in order to include or exclude dung removal activities by the distinguished functional groups (Appendix, Figure A 7.4). Each experimental unit had a square ground surface of 40 cm by 40 cm and walls were 15 cm high. Ground screens were used to exclude tunneler activity, while vertical walls prevented dung removal by rolling species. Ground screens and walls were made of plastic

mesh[4], of which the mesh size determined the size class of beetles that were allowed to enter the experimental unit: 1 cm² to include small beetles, but to exclude large beetles, and 1 mm² to exclude all beetle activity. In case no ground screen or wall was used, respectively tunnelers and rollers of all size classes were able to move dung. As dwellers do not relocate dung, dung removal by this group could only be prevented by excluding all dung beetle activity in a combination of walls and a ceiling in fine mesh material or by combining a fine-meshed ground screen, walls and a ceiling (respectively treatments 10 and 11 in Appendix, Figure A 7.4). In treatments using a coarse meshed ground screen or no ground screen, the dung removing activities of ground fauna other than dung beetles and unable to move through 1 mm² mesh were inevitably included. Dung removal by the latter was measured in treatment 10 (Appendix, Figure A 7.4). Each type of experimental unit was replicated six times for each dung type, which resulted in a total number of 198 experimental units in each study area (Appendix, Figure A 7.3).

Experimental setup

Before starting the experiment the vegetation within the exclosures was cut to a height of ca. 3 cm in order to enable the construction of the experimental units on a level surface. Fresh dung was collected on site from animals that had not been treated with anthelmintics in at least the preceding 6 weeks. Drug-free dung was preferred as anthelmintics have been proven to alter the attractiveness of dung and to have a negative impact on dung beetles (Holter et al., 1993; Ridsdill-Smith, 1993; Wardhaugh and Mahon, 1991). Once collected, the dung was homogenized and divided into standard amounts of 300 g for cattle dung and 200 g for horse and sheep dung and frozen at -20 °C for at least two days (as in e.g., Slade et al. (2007)) in order to kill all groups of biota that consume a considerable part of dung (e.g., dung beetles, earthworms, fly larvae,…) or interfere with the experiment in a different way (e.g., predatory beetles hunting for fly or dung beetle larvae). Mericarps of *Galium aparine* and

[4] Both mesh types were made of sturdy plastic, more specifically polypropylene for the 1 mm² mesh and HDPE (high density polypropylene) for the 1 cm² mesh. Both mesh types were purchased at Conwed plastics (www.conwedplastics.com).

caryopses of *Alopecurus myosuroides* and *Poa pratensis* were used as proxies in the seed dispersal experiment. This selection of species was based on their differing shape and size, which is elongated and large (6 mm), spherical and medium-sized (3 mm) and elongated and small (1 mm) for *A. myosuroides*, *G. aparine,* and *P. pratensis* respectively. All seeds were purchased in a specialized seed web shop (www.herbiseed.com). In order to avoid seed loss caused by germinating seeds during the experiment, seeds were, prior to the experiment, sterilized by dry heating at 80 °C for 7 days. As after 60 days, no seedlings emerged in the subsequent germinability test on 1 % water agar in laboratory conditions, we assumed that no germination during the field experiment would occur. Each seed species was spray-painted in distinct fluorescent colours in order to increase visibility. Ten seeds of each species were mixed with the homogenized dung portions (either cattle dung (300 g), horse dung (200 g) or sheep dung (200 g)).

At the start of the experiment, 1 dung portion was put in the centre of each experimental unit and left on site for 4 weeks. The dung removal experiment was replicated in 2 seasons, summer and autumn, with July 30th 2014 and September 16th 2014 as the respective starting dates[5]. The seed dispersal experiment was conducted once, simultaneously with the dung removal experiment in summer.

Dung beetle sampling

During each experimental run, the dung beetle assemblage was sampled in each study area. To achieve a complete view on dung beetle diversity, two types of pitfall traps were used (Appendix, Figure A 7.5). The first trap type consists of one large 1 l container covered by hexagonal chicken wire (mesh size 25 mm) and a nylon bag filled with dung in the middle (Larsen and Forsyth, 2005). The second trap type consists of five smaller 0.2 l containers

[5] According to the Belgian royal meteorological institute (KMI) **August 2014** had an abnormally low average temperature (16.2 °C versus 18.0 °C) and sunshine duration (149:49 h versus 189:32 h) and abnormally high precipitation (136.0 mm versus 79.3 mm). **September 2014** had an abnormally low precipitation (15.1 mm versus 68.9 mm), high average temperature (16.5 °C versus 14.9 °C) and normal sunshine duration (156:59 h). **October 2014** was characterized by abnormally high average temperatures (13.6 °C versus 11.1 °C), and normal precipitation (58.1 mm) and sunshine duration (97:39 h) (source: www.meteo.be accessed January 2017).

surrounding a central dung pile of approximately 300 g (D'hondt et al., 2008). In both cases, 2 stacked containers were used to ease the emptying, and the upper rim of the containers was levelled with the soil surface. A saturated water-salt solution (365 g/l NaCl with some drops of unscented detergent) was used as fixation fluid. The traps were placed randomly between the experimental units in a randomized pattern with six replicates per trap type (Appendix, Figure A 7.3). As the dung beetle diversity and activity is known to differ between dung types (Finn and Giller, 2002), each trap was baited with one of the used dung types in the experiments: cattle, horses, and sheep. In order to avoid interference with the dung and seed removal experiments, traps were put in operation one week after the start of the experiment. All traps were emptied weekly during the experiments in order to measure dung beetle abundance and diversity with ageing dung. Scarabaeoidea species were identified using Janssens (1960), Jessop (1986) and Baraud (1992). Five specimens of each dung beetle species were dried at 60 °C for 5 days in order to measure average dry biomass. As some members of the Hydrophilidae (mainly *Sphaeridium* and *Cercyon* species) are commonly found in dung in temperate regions (Finn et al., 1999), the number of individuals in this group was counted as an estimate of their abundance. However, as species in this group are not coprophagous during their entire life cycle and do not move dung laterally or vertically, this group was further not considered in the analyses and dung beetles were strictly defined as beetles belonging to the Scarabaeidae and Geotrupidae families. Dung beetle assemblage was defined by species diversity (number of species), total abundance and Shannon diversity index (Jost, 2006). Total biomass was calculated per dung type, experimental period and study site by multiplying and summating species abundance and specific biomass.

Measurements

Of each dung type, three reference samples were taken from the same dung batch that was used in the experiments. The fresh reference samples were oven-dried at 60 °C and their dry weight was subsequently measured for use as a proxy of the wet: dry ratio of fresh dung. At the end of each experimental period, the remaining dung in the experimental units was collected and oven-dried at 60 °C. Once completely dry, each sample's weight was recorded and the amount of seeds left in the summer samples

was counted. To facilitate the counting, samples were pulverized using a blender in short pulses during 30 s. UV light was used to visualize the fluorescent seeds among the dung particles.

Dung removal was calculated as:

$$M_{removed} = \frac{M_{reference} - M_{sample}}{M_{reference}} \; x \; 100$$

where $M_{reference}$ is the average dry mass of the reference samples put in the units at the start of the experiment and M_{sample} is the dry mass of the remaining dung at the end of the experiment. Seed dispersal was expressed similarly as:

$$S_{dispersed} = \frac{S_{initial} - S_{retrieved}}{S_{initial}} \; x \; 100$$

where $S_{initial}$ is the number of seeds put in the dung samples and $S_{retrieved}$ is the number of seeds retrieved from the samples at the end of the experiment.

Statistics

All statistical analyses were performed in R version 3.2.2 (R Core Team, 2015a). Dung preference for each individual species was specified using chi-squared tests for each season and study site. Species diversity and abundance data were analysed in relation to dung age using generalized mixed-effects models (GLMMs, Zuur et al. (2009)) with dung type, trap type, season, study site and dung age as fixed effects and a nested trap location : trap type term as random effect. Species diversity was fitted using the glmer function in the lme4 package (Bates et al., 2014) with Poisson error structure. The abundance count data were over-dispersed and were therefore fitted with a quasi-Poisson error structure using the glmmPQL function in the MASS package (Venables and Ripley, 2002). Biomass was similarly analysed using a linear mixed-effects model with the lme function in the nlme package (Pinheiro et al., 2015). Prior to the analyses, biomass values were square root transformed in order to meet the requirements of normal distribution of the residuals. Models were built for the entire dung

beetle assemblage, and each of the functional groups. The resulting models were simplified using a backward selection process in which covariates were eliminated based on AIC values (Bolker et al., 2009). Significant differences between dung types were determined using Tukey posthoc tests with package multcomp (Hothorn et al., 2008).

As no rollers were found and treatments that differ only in the inclusion or exclusion of rollers did not differ significantly ($F_{4,6}$= 0.636, p=0.701), the number of treatment classes was reduced to five classes: complete assemblage (complete), complete assemblage with the exclusion of large tunnelers (complete-T), dwellers (dwellers), soil fauna (soilF) and negative control (control) (Table 7.1). Differences between treatments, study sites, dung types and seasons were analysed using ANOVA and Tukey posthoc tests. Transformations were applied to dung removal and seed dispersal ratios in order to meet the requirements for parametric tests (*log(x+1)* for dung removal, large seed, and small seed dispersal; *arcsine square root* for medium seeds).

We used hierarchical partitioning (Chevan and Sutherland, 1991) to examine the independent effect of each functional group on dung removal and seed dispersal. Hierarchical partitioning is a multiple regression technique in which all possible models are jointly considered in an attempt to identify the most likely causal factors (Chevan and Sutherland, 1991; Mac Nally, 2000). A binomial error structure was implemented in the models and significance levels were achieved after running randomization tests with 1000 iterations. Hierarchical partitioning and randomization tests were implemented using the hier.part package in the R environment (Walsh and Mac Nally, 2013). The relation between seed dispersal and dung removal was assessed by a multiple regression including seed sizes and study sites.

Table 7.1 - Treatments defined in the dung removal and seed dispersal experiments, with the functional groups able to process dung, the experimental unit types and the number of replicates for each treatment using 1 dung type (*N*). The inclusion or exclusion of functional groups is marked with respectively + and -. A full description of the experimental unit types can be found in Appendix, Figure A 7.4.

	functional groups					
treatment	**dwellers**	**large tunnelers**	**small tunnelers**	**soil fauna**	**experimental unit type**	***N***
complete	+	+	+	+	1, 4, 5	18
complete-T	+	-	+	+	3, 7, 9	18
dwellers	+	-	-	-	2, 6, 8	18
soilF	-	-	-	+	10	6
control	-	-	-	-	11	6

Table 7.2 - Total number of individuals of each species sampled by study area, season and used dung bait. Functional groups (FG) are coded as follows: D= dwellers, T= large tunnelers, t= small tunnelers, O= other beetle groups. Asterisks indicate significant differences between dung types after chi-square tests with ***: $p<0.001$, **: $0.001<p<0.010$, *: $0.010<p<0.050$.

sampled species	FG	individual biomass (mg)	Kalmthout summer		
			C	H	S
Geotrupidae Latreille, 1802					
Geotrupes spiniger (Marsham, 1802)	T	366.2	9	34	19***
Typhaeus typhoeus (Linnaeus, 1758)	T	111.1	0	0	0
Scarabaeidae Latreille, 1802					
Onthophagus coenobita (Herbst, 1783)	t	15.4	0	0	0
Onthophagus similis (Scriba, 1790)	t	4.4	4	60	14***
Acrossus rufipes (Linnaeus, 1758)	D	25.5	29	12	11**
Agrilinus ater (De Geer, 1774)	D	3.2	0	0	0
Aphodius fimetarius (Linnaeus, 1758)	D	9.8	0	0	1
Aphodius foetens (Fabricius, 1787)	D	12.1	4	7	7
Bodilopsis rufa (Moll, 1782)	D	3.1	4	1	12**
Chilothorax distinctus (Müller, 1776)	D	0.1	0	0	0
Melinopterus consputus (Creutzer, 1799)	D	1.6	0	0	0
Melinopterus prodromus (Brahm, 1790)	D	3.0	0	0	0
Melinopterus sphacelatus (Panzer, 1798)	D	2.7	0	0	0
Nimbus contaminatus (Herbst, 1783)	D	2.9	0	0	0
Otophorus haemorrhoidalis (Linnaeus, 1758)	D	2.4	1	0	0
Teuchestes fossor (Linnaeus, 1758)	D	25.7	2	0	2
Volinus sticticus (Panzer, 1798)	D	2.4	0	0	0
Hydrophilidae Latreille, 1802					
Cercyon spec.	O		33	51	40
Sphaeridium spec.	O		1	7	9*
total number[1]			**53**	**114**	**66**
biomass (mg) [1]			**4167**	**13111**	**7484**
species richness[1]			**7**	**5**	**7**
Shannon entropy[1]			**1.414**	**1.149**	**1.703**

[1] calculated using Geotrupidae and Scarabaeidae species only.

Kalmthout			**Zwin**					
autumn			**summer**			**autumn**		
C	**H**	**S**	**C**	**H**	**S**	**C**	**H**	**S**
3	21	25***	1	3	1	3	3	0
149	45	79***	0	0	0	0	0	0
0	0	0	0	0	1	0	0	1
4	15	9*	6	4	2	0	9	7*
1	2	11**	17	0	17***	20	9	28**
0	0	0	1	0	0	0	0	0
3	0	0*	1	0	1	0	0	0
2	3	1	7	9	7	2	8	1*
0	0	7***	8	0	17***	18	0	18**
0	4	2	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1
132	1152	363***	0	0	0	16	28	45***
4	15	3**	0	0	0	9	31	28**
116	4161	386***	0	0	0	49	211	175***
0	0	0	10	0	7***	2	0	6*
0	0	0	4	0	3	0	0	0
1	6	2	2	10	4*	3	8	3
71	30	64***	52	59	62	15	6	14
4	13	8	31	0	3***	0	7	1**
415	**5424**	**888**	**57**	**26**	**60**	**122**	**307**	**313**
18486	**28281**	**20482**	**1079**	**1249**	**1074**	**1913**	**2257**	**1562**
10	**10**	**11**	**10**	**4**	**10**	**9**	**8**	**11**
1.304	**0.646**	**1.237**	**1.953**	**1.272**	**1.864**	**1.721**	**1.150**	**1.460**

Results

Dung beetle fauna

In total 7845 dung beetles were sampled representing 17 species, of which 2 species were classified as large tunnelers, 2 species as small tunnelers and 13 species as dwellers (Table 7.2). No rollers were found at either study site. The large tunnelling species *Typhaeus typhoeus* was found exclusively in Kalmthout in relatively large numbers during the autumn experiment in cattle and sheep dung. In Kalmthout, the relative abundance of functional groups largely varies between seasons. In summer, 60% of the sampled dung beetles are tunnelers while this number drops to 5% in autumn. Nevertheless, the increase in dwellers during the autumn experiment in Kalmthout can almost entirely be attributed to *Nimbus contaminatus* and *Melinopterus prodromus* which make up respectively 73 and 26% of the total number of dwellers sampled in autumn (Table 7.2). Some of the rare species were found exclusively in The Zwin (*Onthophagus coenobita* (2), *Agrilinus ater* (1) and *Melinopterus consputus* (1)) or Kalmthout (*Chilothorax distinctus* (6)). More species were found during the autumn experiment (*Wald Z-test: z*= 0.082, *p*<0.001). *Typhaeus typhoeus*, *Chilothorax distinctus*, *Melinopterus consputus*, *Melinopterus sphacelatus*, *Melinopterus prodromus* and *Nimbus contaminatus* were trapped exclusively in autumn (Table 7.2). For *M. prodromus* and *N. contaminatus*, we collected the largest number of individuals: 1736 and 5098 respectively.

Overall, more dung beetle species were found in The Zwin (*Wald Z-test: z*= -3.512, *p*<0.001), although dung beetles were collected more abundantly in Kalmthout (*Wald t-test: t*= -6.630, *p*<0.001) and especially in autumn (*Wald t-test: t*= -8.019, *p*<0.001) (Table 7.3). Similarly, more biomass of both dweller and tunneler species was collected in Kalmthout (*Wald t-test: t*= -11.014, *p*<0.001) and during the autumn experiment (*Wald t-test: t*= -7.683, *p*<0.001). None of the used dung types had a significant effect on tunneler diversity, while more dweller species were found in sheep dung compared to cattle dung (*Wald Z-test: z*= 2.567, *p*= 0.028). Dung beetles clearly preferred horse dung over cattle (*Wald Z-test: z*= -6.443, *p*<0.001) or sheep dung (*Wald Z-test: z*= -5.490, *p*<0.001). Overall, a higher abundance and diversity of dwellers was found and both diversity and abundance decreased with ageing dung for all considered functional groups (Figure 7.1).

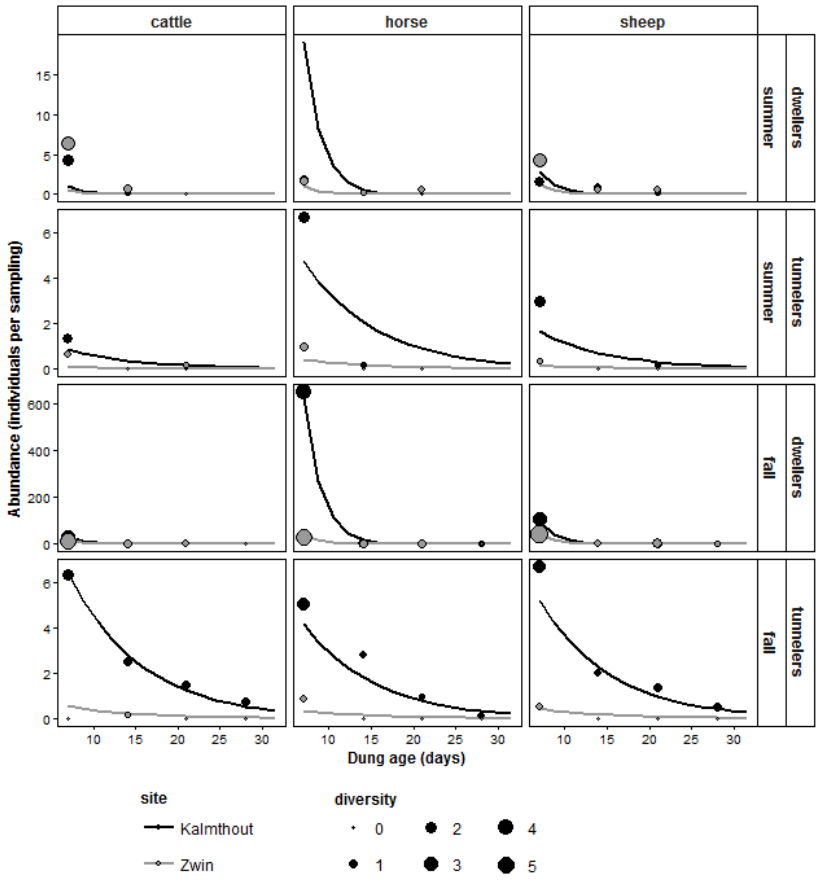

Figure 7.1 - Abundance (number of individuals per sampling) in relation to dung age by functional groups (tunnelers and dwellers) and by dung type (cattle, horse and sheep) in summer and autumn. Symbol sizes reflect species richness. Lines are fitted from GLMM's with trap location and trap type as random effect.

Table 7.3 - Results of the generalized linear mixed-effects models (GLMM) with diversity and abundance as response variables, and the linear mixed-effects model with biomass as response variable. In each model the nested term trap location: trap type was used as random effect.

			diversity		abundance		biomass	
functional group	**fixed effects**	***df***	**Wald χ^2**	***p***	**Wald χ^2**	***p***	**Wald χ^2**	***p***
all	dung age	1	396.891	<0.001	90.485	<0.001	177.694	<0.001
	study site	1	12.171	<0.002	40.580	<0.001	124.019	<0.001
	trap type	1	12.426	<0.003	20.582	<0.001	3.210	0.073
	season	1	90.301	<0.004	49.726	<0.001	60.346	<0.001
	dung type	2	7.362	0.025	59.216	<0.001	0.880	0.644
	study site*:* dung type	2	2.646	0.266	11.048	0.004	0.799	0.671
	season*:* dung type	2	3.855	0.146	5.667	0.059	1.267	0.531
	trap type*:* dung type	2	6.651	0.036	0.513	0.774	1.052	0.591
tunnelers	dung age	1	76.488	<0.001	125.586	<0.001	78.434	<0.001
	study site	1	80.462	<0.001	97.665	<0.001	140.675	<0.001
	trap type	1	16.622	<0.001	0.094	0.759	0.237	0.627
	season	1	0.166	0.684	20.707	<0.001	32.965	<0.001
	dung type	2	1.764	0.414	2.032	0.362	0.010	0.995
	study site*:* dung type	2	0.256	0.880	0.820	0.664	0.776	0.678
	season*:* dung type	2	3.861	0.145	36.106	<0.001	8.826	0.012
	trap type*:* dung type	2	1.544	0.462	0.545	0.762	1.361	0.506

Table 7.3 continued.

			diversity		abundance		biomass	
functional group	**fixed effects**	***df***	**Wald χ^2**	***p***	**Wald χ^2**	***p***	**Wald χ^2**	***p***
dwellers	dung age	1	313.053	<0.001	32.591	<0.001	547.605	<0.001
	study site	1	6.143	0.013	19.722	<0.001	3.092	0.079
	trap type	1	65.828	<0.001	15.311	<0.001	10.885	<0.001
	season	1	15.537	<0.001	14.072	<0.001	92.347	<0.001
	dung type	2	6.154	0.046	37.233	<0.001	9.286	0.010
	study site*:* dung type	2	2.990	0.224	7.648	0.022	9.001	0.011
	season*:* dung type	2	14.623	<0.001	3.434	0.180	21.034	<0.001
	trap type*:* dung type	2	5.396	0.067	0.339	0.844	1.717	0.424

Table 7.4 - ANOVA results for the removal of dung and dispersal of large, medium and small seeds.

		dung removal		large seeds		medium seeds		small seeds	
factor	***df***	***F* value**	***p***	***F* value**	***p***	***F* value**	***p***	***F* value**	***p***
study site	1	155.307	<0.001	60.313	<0.001	212.062	<0.001	127.33	<0.001
season	1	21.817	<0.001						
dung type	2	7.712	<0.001	14.139	<0.001	18.408	<0.001	3.200	0.042
treatment	4	64.342	<0.001	24.870	<0.001	22.093	<0.001	5.483	<0.001
study site x season	1	13.118	<0.001						
study site x dung type	2	9.446	<0.001	4.551	0.011	20.772	<0.001	16.24	<0.001
dung type x season	2	15.307	<0.001						
study site x treatment	4	9.887	<0.001	3.635	0.006	4.572	0.001	4.600	0.001
dung type x treatment	8	0.940	0.482	0.618	0.763	1.874	0.062	1.638	0.113

Table 7.5- Average values and standard errors for dung removal (%) and seed dispersal (%) by study site and dung type. Different letters in the same column indicate significant differences between treatments within each measured variable and season (ANOVA and Tukey posthoc tests). Asterisks indicate significant differences between sites (column measured variable) or seasons (column season).

measured variable	season	treatment	Kalmthout			Zwin		
			cattle	horse	sheep	cattle	horse	sheep
dung removal*	summer*	complete	29.1±3.0a	46.2±4.2q	44.9±3.6x	87.8±1.5a	78.4±2.6q	60.7±3.9x
		complete-T	34.2±1.9a	38.0±2.5q	42.7±3.2x	46.5±3.9b	64.2±2.2r	50.7±2.5xy
		dwellers	28.0±3.0a	41.6±0.7q	41.3±0.9x	26.6±2.4c	45.6±2.6s	38.2±2.7y
		soilF	27.2±3.9a	42.0±1.6q	47.0±5.8x	76.7±9.4a	77.6±3.5q	57.3±8.0x
		control	32.0±2.8a	41.2±1.8q	43.1±1.7x	21.7±4.5c	41.9±1.8s	34.5±5.0y
	autumn*	complete	53.6±7.6a	46.9±5.0q	48.1±4.9x	53.8±1.5a	52.1±1.8q	51.9±2.3x
		complete-T	35.0±1.7ab	31.6±1.7qr	32.9±2.0y	46.9±1.8b	48.4±1.3qr	45.7±1.0xy
		dwellers	36.0±2.1ab	28.8±1.3r	28.9±2.5y	38.0±0.8c	42.8±1.5rs	34.9±1.3z
		soilF	51.2±12.9ab	50.0±9.7q	64.9±10.4x	50.3±2.8ab	50.0±2.0qr	46.0±3.3xy
		control	26.1±1.5b	21.5±2.0r	21.4±1.3y	38.0±1.6c	37.5±0.8s	35.4±4.8yz
large seeds*	summer	complete	17.8±3.6a	45.0±6.9q	48.8±6.2x	72.2±6.2a	66.7±5.8q	56.7±6.0x
		complete-T	20.6±5.1a	42.8±4.9q	45.6±6.4x	43.9±6.8bc	57.2±5.9q	55.6±6.1x
		dwellers	17.2±3.1a	22.8±3.3r	27.6±4.2x	20.7±3.2bc	30.6±5.8r	36.7±5.0x
		soilF	23.3±6.1a	26.0±4.0qr	40.0±8.6x	53.3±12.8ab	70.0±8.9q	56.0±4.0x
		control	13.3±5.6a	6.0±4.0r	28.3±7.5x	13.3±5.6c	30.0±5.2r	28.3±8.7x

Table 7.5 continued.

measured variable	season	treatment	Kalmthout			Zwin		
			cattle	horse	sheep	cattle	horse	sheep
medium seeds*	summer	complete	22.8±4.1a	60.6±6.1q	50.6±6.4x	95.6±1.5a	94.4±2.3q	63.9±6.1x
		complete-T	15.6±2.0a	45.6±6.2qr	43.1±7.1x	69.4±4.7b	83.3±3.7qr	64.4±5.7x
		dwellers	17.8±3.0a	36.7±4.6r	33.5±4.4x	41.3±7.4c	66.1±7.1rs	51.7±6.5x
		soilF	28.3±6.0a	30.0±11.4r	48.3±10.1x	90.0±10.0ab	91.7±3.1qr	72.0±12.4x
		control	15.0±3.4a	28.0±4.9r	30.0±8.6x	60.0±12.4bc	41.7±17.0s	31.7±9.8x
small seeds*	summer	complete	29.4±4.3a	50.6±6.7q	65.0±6.2x	85.0±4.0a	86.7±4.2q	71.7±5.1x
		complete-T	38.9±3.6ab	46.1±4.4qr	59.4±3.8x	78.9±6.1ab	77.2±5.0q	70.6±4.7x
		dwellers	33.9±4.3ab	47.2±4.6qr	52.9±6.3x	60.7±6.9b	56.1±6.2r	56.1±5.4x
		soilF	36.7±6.7ab	16.0±2.4r	58.3±4.8x	75.0±14.5ab	90.0±4.5q	72.0±9.2x
		control	55.0±4.3b	44.0±9.8qr	45.0±6.2x	76.7±6.1ab	46.7±6.1r	60.0±8.9x

Dung removal and seed dispersal

On average, more dung was removed in The Zwin than in Kalmthout, especially in summer (Table 7.4 and Table 7.5). In The Zwin significantly more dung was removed during the summer experiments while in Kalmthout no seasonal differences were found. In summer, more horse dung was removed compared to cattle and sheep dung in The Zwin, while significantly more sheep dung was removed in Kalmthout. In both areas, significant differences between treatments were found (Table 7.4). Dung removal by soil fauna was significant in The Zwin (summer and autumn) and in Kalmthout during the autumn experiment (Figure 7.2). Large tunnelers removed a large amount of dung of all tested dung types in Kalmthout during the autumn experiment, while this effect was only significant for horse dung in the summer experiment. In The Zwin large tunnelers removed more dung during the summer experiment; while in the autumn experiment both large and small tunnelers were equally important dung removers. In both study sites and seasons, the contribution of dwellers in dung removal was insignificantly small (Figure 7.2). Seed removal was positively correlated with dung removal, although differences between study sites and seed sizes were found (Table 7.4 and Table 7.6). More large, medium and small seeds were removed in The Zwin compared to Kalmthout, which is in line with our results on dung removal (Table 7.5). Large seeds were primarily dispersed by soil fauna other than dung beetles in both study areas (Figure 7.2). Generally, the role of each functional group in seed dispersal was similar to their importance in dung removal. However, dwellers dispersed large amounts of small seeds in cattle and horse dung in Kalmthout, while their effect on dung removal was limited. Also, small tunnelers dispersed more large seeds compared to large tunnelers in Kalmthout, while the opposite was found in dung removal (Figure 7.2).

Table 7.6 - Regression coefficients of the multiple regression between seed dispersal and dung removal by seed size and study site ($F_{5,1150}$ = 1535, p<0.001, R^2=0.854).

factor	estimate±SE	*t* value	*p*
dung removal	0.70±0.04	19.341	<0.001
large seeds	-0.84±1.91	-0.439	0.660
medium seeds	12.33±1.91	6.441	<0.001
small seeds	18.80±1.91	9.820	<0.001
site Zwin	14.02±1.45	9.689	<0.001

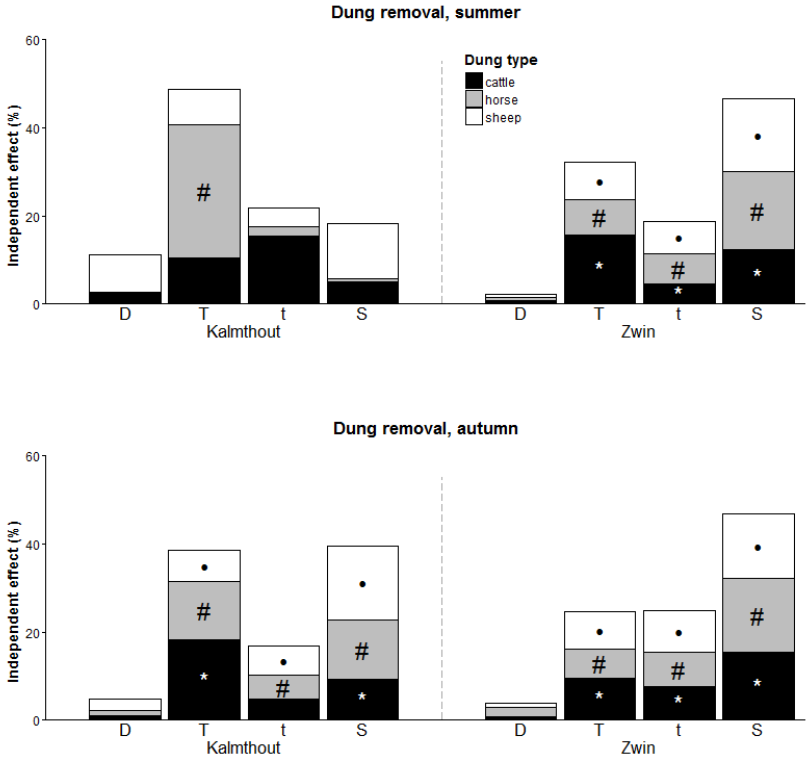

Figure 7.2 - Hierarchical partitioning results for dung removal (summer and autumn experiment) and dispersal of large, medium and small seeds by each functional group: dwellers (D), large tunnelers (T), small tunnelers (t) and other soil inhabiting fauna (S). The percentage of independent effect contributed by each of the functional groups is plotted for the studied dung types. Symbols indicate whether the contribution of each functional group is significant (p<0.050) for cattle (asterisks), horse (hash tags) and sheep dung (filled dots) after evaluation of the Z-scores of randomization tests with 1000 iterations.

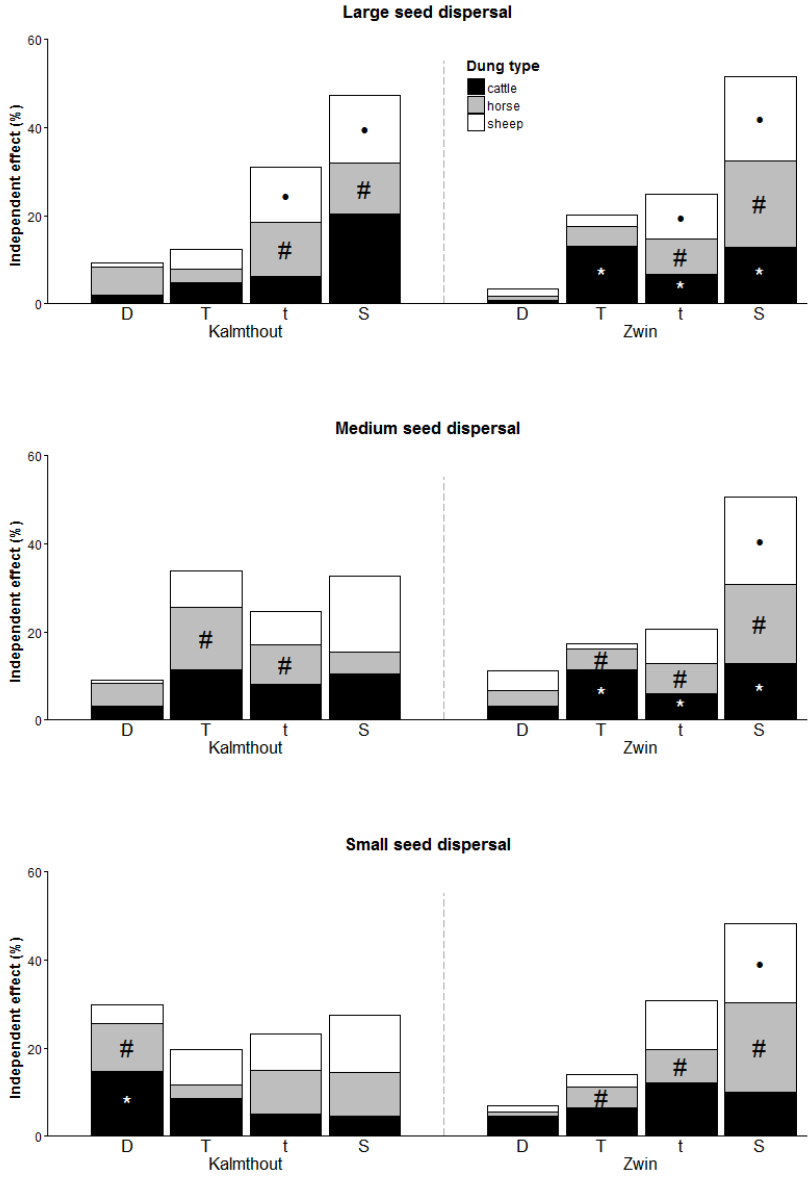

Figure 7.2 continued.

Discussion

Dung beetle diversity and abundance

Overall, dung beetle assemblages in both study sites were dominated by dweller species which corresponds to what is expected at this latitude. The functional composition of dung beetle assemblages is known to vary between biogeographical regions with a shift from dweller dominated assemblages in the northern climatic zones towards a dominance of larger tunnelers and rollers in southern biogeographical regions (Hanski and Cambefort, 1991b; Hortal et al., 2011; Verdú and Lobo, 2008). *M. prodromus*, *M. sphacelatus* (Gittings and Giller, 1997) and *N. contaminatus* (Hanski, 1980) generally do not breed in dung. Still, as in our study and previous studies (Finn et al., 1999; Hutton and Giller, 2003) these species with generalist saprophagous larvae were found abundantly in dung baited traps, dung may be an important food source for the imagos of these species. Mass occurrences of generalist dweller species such as *M. prodromus* and *N. contaminatus* in our study have been reported widely in northern temperate ecosystems, but it seems to occur infrequently (Finn and Gittings, 2003; Hanski and Cambefort, 1991b; Holter, 1982). Although mass occurrence events certainly lead to reproduction failure in other dweller species due to the rapid dung decomposition associated with these events, the mechanism of mass occurrences is not yet revealed (Finn and Gittings, 2003). We should also keep in mind that relatively large abundances of *Cercyon* and *Sphaeridium* species have been trapped which suggests that these species have their share in dung removal. Despite the fact that the larvae of these Hydrophilids are predators on fly larvae in dung (Hanski and Koskela, 1979; Sowig, 1997), adults have a similar feeding strategy as dung beetles (Holter, 2004) and oviposit in dung, which makes them functionally equivalent to dwelling dung beetles sensu stricto. As our experimental setup did not allow for discrimination between the activity of Hydrophilidae and Scarabaeidae species, ecological functions provided by dwellers should be interpreted as the sum of the action of both groups.

Dung as a food source

Dung beetles clearly prefer fresh dung as both the diversity and the abundance of the sampled dung beetles rapidly declines after the first week. Previous research in the temperate climate zone also found a clear preference for fresh dung (Finn and Giller, 2002) which might be related to the loss of scent (Dormont et al., 2004; Dormont et al., 2007) and water content (Holter and Scholtz, 2007) of older dung. Our results suggest that tunnelers remove most dung in the first days following dung deposition and dwellers make chambers and oviposit in fresh dung pats.

Furthermore, most species prefer a certain type of dung, but this effect is not always straightforward when comparing seasons and study sites. In most cases species either prefer dung from hindgut fermenters (horses) or ruminants (cattle and sheep). This preference can be addressed to the different consistency of both dung types as dung of hindgut fermenters mostly consists of light, uncompressed dung containing large plant particles (Steuer et al., 2013) while ruminant dung has a smaller average particle size and is much denser. As 40-50% of the dry fraction of fresh cattle, horse and sheep dung contains particles small enough for dung beetle ingestion (Holter, 2000), the preference for a certain dung type is most likely not driven by a shortage of food resources but rather by nesting strategies. Therefore, the use of different herbivore species in nature management results in more diverse dung beetle assemblages, but on the other hand, reduced dung beetle diversity may lead to slower dung degradation as certain specialized species might be missing.

Top: cattle dung pat with tunnels (autumn 2015, Kalmthout, Belgium). Bottom: *Typhaeus typhoeus*, the most abundant large tunneler in autumn at the study site in Kalmthout. ©Bastiaan Notebaert

Dung removal rate

Tunnelers, and especially large tunnelers, removed a large proportion of dung whereas dwellers had no significant effect on dung removal. In addition to dung beetles, the presence of epigeic earthworms, dung flies and their combinations can largely alter the decomposition of dung (O'Hea et al., 2010). While the dung removing activity of coprophagous dipteran larvae resembles the behaviour of dwellers, earthworms actively move dung through tunnels beneath dung pats which is comparable to the action of tunnelers. Our set-up did not allow differentiating dung removal by dwellers and fly larvae, but as in treatment soilF only digging macro-invertebrates were able to reach the dung pat, we made an estimate of the impact of this group. In both areas, digging soil macro-invertebrates other than dung beetles were important dung removers. According to Gittings et al. (1994) and Gittings and Giller (1999) other soil-inhabiting macro-invertebrates, such as earthworms, can fulfil an important role in dung decomposition in northern Europe. In an extensive review of competitive interactions between dung fauna, Finn and Gittings (2003) also classified earthworms as a separate functional group for which dung is an important food source in both the adult and larval stage. Especially in cool, wet weather, earthworms can even remove an entire dung pat before dweller larvae have completed their development (Gittings and Giller, 1999). Although we did not quantify them, earthworms were indeed abundantly present in dung at both study sites. The high dung removal in the treatments in which only soil organisms acted as dung manipulating fauna suggests that earthworms fulfil an equal role in dung removal as tunnelling dung beetles. The fact that equal portions of dung are removed by earthworms alone and the complete functional assemblage suggests that competition takes place between large tunnelers and earthworms and opposes the findings of Holter (1983) who concludes that dung with beetles is more attractive to earthworms than dung without dung beetles. On the other hand, Rosenlew and Roslin (2008) conclude that large tunnelers exceed earthworms in dung removal. In our study, large tunnelers were important dung removers as well, but the amount of dung removed by this group differs between sites and seasons. Although large tunnelers were clearly more abundantly present in Kalmthout, dung removal by this group was lower but still rather high in the Zwin. Furthermore, dung beetle biomass is relatively high at both sites which is in line with the positive correlation between tunneler biomass and dung

removal found earlier (Horgan, 2005) and which is most apparent for large fast-burying tunnelers on sandy soils (Davis, 1996) such as our study sites.

Secondary seed dispersal

In tropical biomes dung beetles generally do not treat dung with seeds differently than seed-free dung, given that the seeds are relatively small in comparison to the beetle (Andresen, 2002a; Braga et al., 2013; Slade et al., 2007). Our results suggest similar behaviour of dung beetles in temperate ecosystems as a clear linear relation between dung removal and seed dispersal was found, although this was not the case for all seed size classes. Tunnelers and soil fauna are the main dispersers of large and medium seeds, while these patterns were less apparent for the smallest seed class. As high removal rates were found in the control treatments and the treatments including dwellers which are not known to move dung, these results should be interpreted with caution. Despite the efforts put in the recollection of seeds, it is probable that a portion of seeds was overlooked or lost due to other reasons, such as seed loss following heavy rain. Using a larger amount of seeds as a proxy could overcome this problem. Unlike in the tropics, dung beetle assemblages in the northern temperate region are mainly composed of dwelling and tunnelling species and generally lack rolling species. As a result, seeds are almost exclusively dispersed in a vertical direction instead of the combination of horizontal and vertical dispersal in the case of roller presence. Seed burial is often considered beneficial for seed survival as it is hidden for seed predators such as rodents (Shepherd and Chapman, 1998). On the other hand, germination and establishment probabilities decrease when seeds are buried too deep. Unlike the numerous examples of positive effects of dung beetle activity on seed germination and seedling establishment in the tropics (e.g., Andresen and Levey (2004), Shepherd and Chapman (1998)), D'hondt et al. (2008) found a negative correlation in a coastal grassland in the northern temperate zone; they ascribe this to large tunnelling species that bury seeds too deep. As also earthworms are known to dig deep (Ojha and Devkota, 2014) and body size and tunnel depth are positively correlated in tunnelers (Gregory et al., 2015), seedling establishment could be similarly affected as in the study of D'hondt et al. (2008).

We conclude that ecological functions performed by dung beetles are of great importance in northern temperate ecosystems and differ between functional groups. Although more dweller species were found, large tunnelers are disproportionally important in dung burial and seed removal. The dung decomposing activities of small tunnelers and other soil inhabiting fauna, such as earthworms, is of prime importance as well, which stresses the need for conservation of functionally complete dung ecosystems.

Acknowledgments

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Appendix

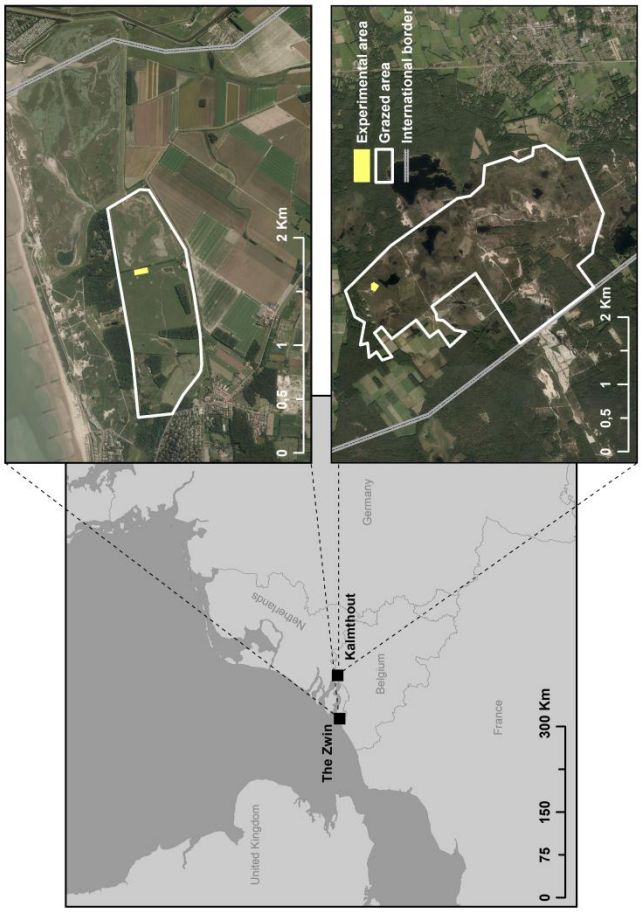


Figure A 7.1 - Location of study areas within Belgium (left) and the extent of the grazed areas and the positioning of the experimental locations in the 'Zwindunes and Zwinpolders' (Zwin, top right) and 'De Zoom - Kalmthoutse heide' (Kalmthout, bottom right). The base layers used in the detailed maps are aerial photographs of summer 2012 provided by the Flanders Geographical Information Agency. © AGIV (http://www.agiv.be/)

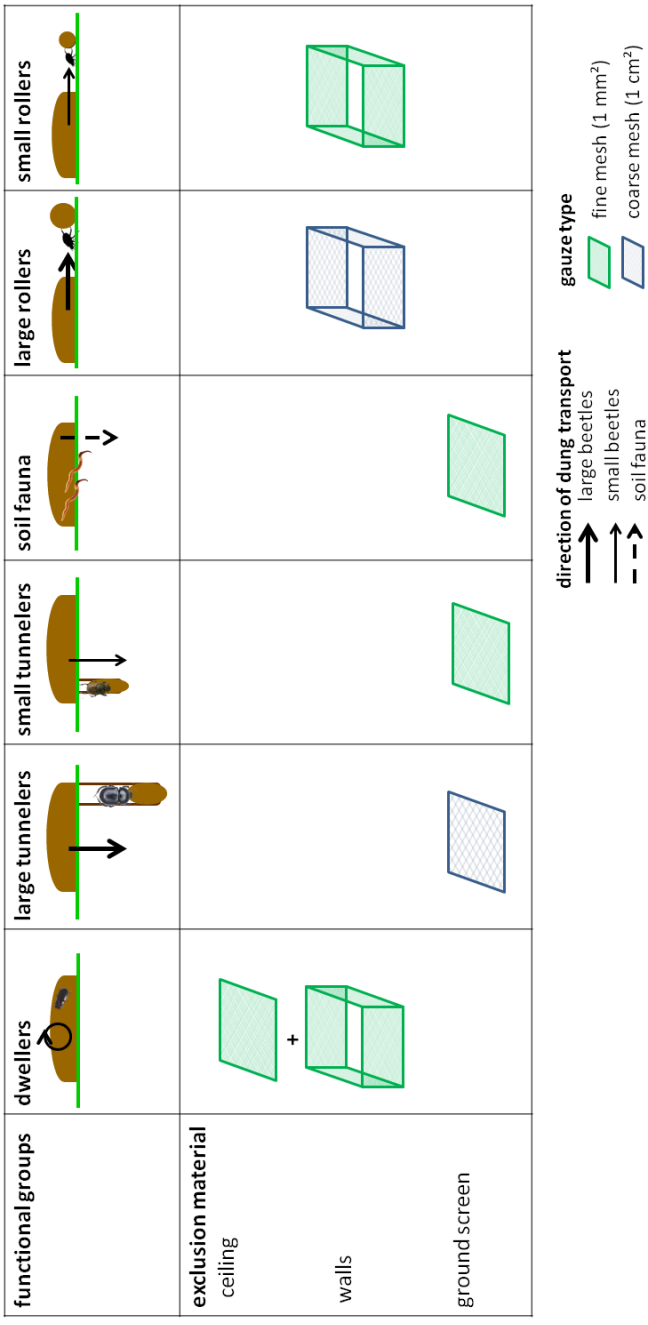


Figure A 7.2 - Functional groups distinguished in the experiments, direction of dung transported by each group and type of material used to exclude their dung removing activities.

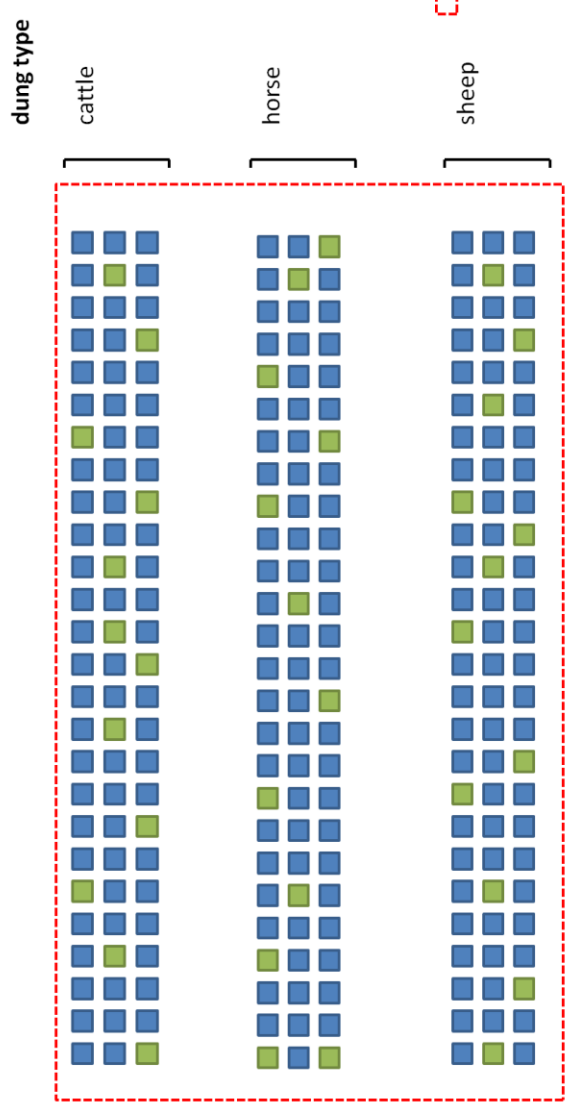


Figure A 7.3 - Experimental design in an exclosure with a randomized distribution of experimental units and sampling units in different sections baited with different dung types.

Experimental units

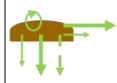
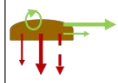
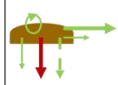
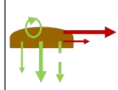
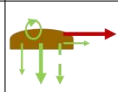
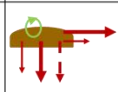
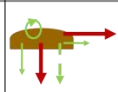
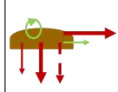
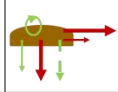
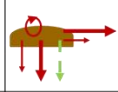
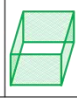
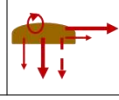
	functional groups included	ground screen	wall	ceiling	dung transport (→ direction prohibited; → direction allowed)
1	all: dwellers tunnelers (l+s) rollers (l + s) soil fauna				
2	dwellers rollers (l + s)				
3	dwellers tunnelers (s) rollers (l + s) soil fauna				
4	dwellers tunnelers (l + s) soil fauna				
5	dwellers tunnelers (l + s) rollers (s) soil fauna				
6	dwellers				
7	dwellers tunnelers (s) rollers (s) soil fauna				
8	dwellers rollers (s)				
9	dwellers tunnelers (s) soil fauna				
10	soil fauna				
11	none (control)				

Figure A 7.4 - Experimental units with the functional groups able remove dung from the units, used materials and direction of dung transport prohibited and allowed (respectively red and green arrows) by dung beetles and other soil fauna (respectively full and dashed lines). Tunnelers and rollers were subdivided into size classes: large beetles (l, stopped by 1 cm² mesh) and small beetles (s, able to go through 1 cm² mesh).

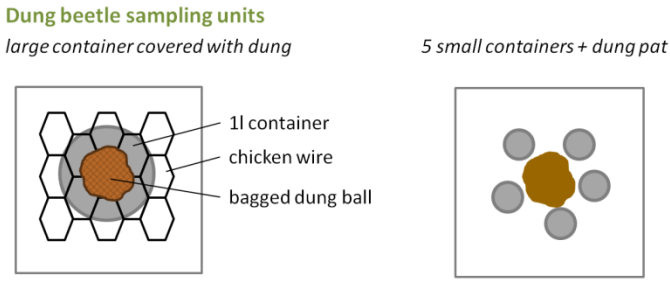


Figure A 7.5 - Schematic top view of the sampling units consisting of 1 large container covered with dung of 5 small containers surrounding a central dung pat.

8 Dung removal and secondary seed dispersal by different dung beetle assemblages along a biogeographic and climatologic gradient: a multi-site experiment in the Western Palaearctic

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Scarabaeus spec. rolling a dung ball at Parco Naturale Migliarino San Rossore Massaciuccoli, Italy (August 2015).

Abstract

Dung beetles are a globally distributed insect group and fulfil a similar role in various terrestrial ecosystems, biogeographic regions and climate zones. They provide a wide range of ecosystem functions related to their dung manipulating behaviour such as nutrient cycling, soil aeration, parasite suppression, secondary seed dispersal and plant growth.

We conducted a large-scale field experiment at 17 study sites in different biogeographic regions and climate zones within the Western Palaearctic. Dung beetle species were classified into functional groups according to their dung processing behaviour and size: dwellers, small and large tunnelers, and small and large rollers. By constructing different exclosure types that allowed or restricted the dung and seed removing activities of certain functional groups of the local dung beetle assemblage, we estimated the role of each group in the ecosystem functions of dung removal and secondary seed dispersal.

Dung beetle assemblages clearly differed along a north-south and east-west gradient, with higher species and functional group richness and dung beetle abundance at lower latitudes. As expected, northernmost sites were dominated by dwellers while a functional shift towards more tunnelers was found in the southern sites. Rollers were found in some regions albeit with very low abundance and species richness. Higher species richness and dung beetle abundance in the southern sites was associated with higher dung removal rates. Tunnelers and rollers were more effective dung removers than dwellers; other soil macro-invertebrates partially took over the dung removing activities of tunnelers in the dweller dominated northern sites. A positive interaction between functional group richness and dung removal rates was found and consequently stresses the need of functional group conservation in order to maintain the ecosystem functions of dung removal and the hereto linked secondary seed dispersal.

Given the north-south gradient in dung beetle functional group composition and given the known high mobility/dispersal capacity of dung beetles in general, it can be expected that climate change may in time increase dung beetle functional diversity to the north, leading to a change in ecosystem functioning.

Keywords: conservation, dung removal, functional diversity, multi-site experiments, Scarabaeidae, secondary seed dispersal, seed fate, Western Palaearctic

Introduction

Human activities have resulted in loss of biodiversity at a faster pace than the background rates estimated from fossil records (Millennium Ecosystem Assessment, 2005). Extinctions alter key ecological processes and hence affect productivity and sustainability of ecosystems (Hooper et al., 2005; Loreau et al., 2001), disrupt ecosystem functioning (Cardinale et al., 2002; Hooper et al., 2012; Larsen et al., 2005) and threaten human well-being (Cardinale et al., 2012; Díaz et al., 2006). Therefore, the relation between biodiversity and ecosystem functions has been subject of an increasing number of scientific publications in the recent years in an attempt to cope with the future challenges of potentially 'incomplete' ecosystems (e.g., Kremen (2005), Larsen et al. (2005), Millennium Ecosystem Assessment (2005)). Even more important than the loss of a single species is the functional shift in species assemblages: species with particular traits disappear or get replaced by species with different sets of functional traits which in consequence leads to changes in ecosystem functioning and ecological pathways (Barnes et al., 2014; Cardinale et al., 2012; Díaz et al., 2006; Loreau et al., 2001). Many studies highlight the importance of facilitation or niche complementarity and the value of multispecies assemblages for certain ecosystem functions (Cardinale et al., 2002; Schmid et al., 2008). However, many of these studies have been criticized for their artificial and restricted species assemblages under strictly controlled abiotic conditions (Finn, 2001; Naeem and Wright, 2003). Therefore, in addition to these controlled (ex situ) lab experiments, large-scale field studies are needed to investigate the results in a more natural context and will help to verify the predictive ability experimental (environmentally down-scaled) research results (Naeem and Wright, 2003; Slade et al., 2007). Focal taxa are often used as proxies for explaining general patterns in biodiversity research and the link with ecosystem functions. Scarab beetles are an excellent taxonomic group for field studies as they are a diverse group both in terms of species diversity and functional diversity. They have a broad geographic distribution and are present in various habitats. They are generally

considered as mobile insects and expected to be good dispersers (Roslin and Viljanen, 2011), although not that many field data are available (e.g., Roslin (2000), Hanski and Cambefort (1991c)). Furthermore, they can be easily sampled within a wide range of sites using standardized protocols and, therefore provide useful data for comparing levels of biodiversity across multiple spatial scales (Spector, 2006). As most of the ecosystem functions provided by dung beetles are related to the local removal and underground burial of dung (Nichols et al., 2008), their diversity can be manipulated at small spatial scales (Manning et al., 2016; Slade et al., 2007).

A great economic value can be attributed to the ecosystem functions provided by dung beetles (Ridsdill-Smith and Edwards, 2011) such as nutrient cycling (e.g., Sitters et al. (2014)) with increased mineralization of the organic nitrogen contained in dung (Yokoyama et al., 1991), bioturbation and the enhancement of hydrological properties of soils (Brown et al., 2010), the stimulation of plant growth and the improvement of forage quality (Bang et al., 2005b; Bornemissza and Williams, 1970), a reduced transmission of gastrointestinal parasites (Gregory et al., 2015), the reduction of pest flies (Bornemissza, 1970; Walsh and Cordo, 1997), and the decreased emission of the greenhouse gas methane (Penttilä et al., 2013; Slade et al., 2016). In addition to these ecosystem functions with a clear and direct economic return, dung beetles are of great potential relevance in plant dispersal ecology as they facilitate secondary seed dispersal. For tropical ecosystems it has been shown that burial of seeds embedded in mammalian faeces by beetles can have direct implications for the composition and maintenance of plant communities (Andresen, 2002a; Feer, 1999). The dispersal of plant seeds through ingestion by vertebrate animals (endozoochory) and the consecutive deposition within dung is a common dispersal mechanism (Pakeman et al., 2002). Through endozoochory, large quantities of seeds that survived the intestinal tract are dispersed a first time and therefore escape from the parental environment. In the event that organisms such as dung beetles remove dung particles containing seeds, an additional dispersal step is made which directs small numbers of seeds to safe sites (Vander Wall and Longland, 2004). The larvae and adult individuals of dung beetles do not feed on seeds and bury seeds unintentionally (Nichols et al., 2008). By spreading seeds from the original dropping site, the degree of seedling competition decreases (Andresen and Levey, 2004) and the burial of seeds along with dung provides a nutritive though organic

environment for young seedlings. Additionally, seed predation losses are reduced as a consequence of burial (Feer, 1999). On the other hand, germination success typically decreases dramatically with burial depth, suggesting that an optimal burial depth exists, allowing both escape from predation and germination (Shepherd and Chapman, 1998).

Dung beetles can be classified into three main functional groups in terms of dung manipulation and nesting strategies (Doube, 1990): tunnelers (paracoprids) which dig shafts and chambers beneath dung pats and fill them with dung for feeding or breeding; rollers (telocoprids) which make balls of dung, roll them away from the pat and bury it for feeding or breeding; and dwellers (endocoprids) which feed and oviposit in the dung pat or at the dung-soil interphase where the complete larval development takes place. Kleptocoprids who are nest parasites of rollers or tunnelers could be considered as a fourth functional group, but as they do not move dung themselves their role in most abovementioned ecosystem functions is rather limited. At the spatial scale, the functional composition of dung beetle assemblages is highly variable between biogeographic and climatologic zones (e.g., the differentiation found in tropical, subtropical, semi-desert rangeland, warm temperate, cold temperate and boreal ecosystems, and in wet versus dry conditions), habitat, altitude and dung availability (Hanski and Cambefort, 1991b; Menendez and Gutierrez, 1996; Scholtz et al., 2009). Differences are also reported on a smaller geographic scale. A clear shift from dweller dominated assemblages in northern temperate climate zones to increasing numbers of tunnelers and rollers in southern temperate zones can be found (Hanski and Cambefort, 1991b). Furthermore, species richness and ecological strategy of dung beetles differ among climate zones and latitudes. The few species present in northern areas are mostly resource generalists, whereas dung beetle assemblages at lower latitudes, with more species per se, are more variable and contain more resource specialist species (Hortal et al., 2011). As functional group richness and species composition of dung beetle assemblages strongly affect key ecological functions such as dung removal and decomposition (O'Hea et al., 2010; Slade et al., 2007), secondary seed dispersal of seeds already present in dung (Slade et al., 2007) and subsequent seed germination (D'hondt et al., 2008), a spatial variation in these ecosystem functions provided by dung beetles can be expected.

Although dung beetles are a globally distributed insect group fulfilling a similar role in terrestrial ecosystems throughout the world, the majority of the research papers on the role of dung beetles in ecosystem function focus either on tropical and subtropical regions or are conducted in agricultural landscapes or microcosms. Especially studies of the impact of various dung beetle assemblages in semi-natural landscapes are restricted to a continental scale. We conducted a large-scale field experiment at multiple sites in different biogeographic and climate zones in the Western Palaearctic. By establishing different exclosure types that allowed or inhibited the dung and seed removing activities of specific functional groups of the local dung beetle assemblage, we estimated the role of each group in dung removal and secondary seed dispersal. According to the large scale of this study, we hypothesize that the differing dung beetle assemblages as found by Hanski and Cambefort (1991b) with functionally more diverse assemblages in the south has a strong effect on dung and seed removal patterns. Furthermore, we expect to find a dominance of resource generalists in the northern areas and more resource specialists in the south. Therefore, different dung types might attract various dung beetle assemblages, with differences between the dung of foregut and hindgut fermenters and between biogeographic regions. More specifically, we aim to find an answer to the following research questions:

1. Is the local dung beetle assemblage more driven by habitat filters (climate, dung type) or by biogeography (latitude, longitude)?
2. Do functionally rich communities provide significantly better ecosystem functions in removal and seed dispersal?

In this study, we defined 'dung beetles' as species of the superfamily Scarabaeoidea that generally feed on dung in both the larval and adult phase. Some species of other beetle families such as Hydrophilidae and Staphylinidae are commonly found in dung as well and could be considered as dung beetles (Hanski and Cambefort, 1991b). Nevertheless, as they are not coprophagous during their entire life cycle (Finn et al., 1999) and they do not contribute to lateral or vertical dung transport they are not included in this experiment. Therefore, dung beetles were strictly defined as the coprophagous species in the Geotrupidae and Scarabaeidae families.

Material and methods

Study sites

We carried out a multi-site experiment on 17 study sites, covering 10 countries, 11 biogeographic and 6 climate zones within the Western Palaearctic realm (Appendix, Table A 8.1, Figure A 8.1 and Figure A 8.2). In order to build a statistically solid dataset we replicated the experiment on a spatial scale by selecting study sites within the same zone (Appendix, Table A 8.2) and on a temporal scale by repeating the experiment in the summers of 2014, 2015 and/or 2016 (Appendix, Table A 8.2). All study areas consisted of more or less natural grasslands which had been grazed by domestic and/or wild herbivores for at least a couple of years prior to the experiment (Appendix, Table A 8.2 and Table A 8.3). Biogeographic provinces (Udvardy, 1975), Köppen-Geiger climate zones (Peel et al., 2007), and EUNIS habitat types as defined by the European Environmental Agency (http://www.eea.europa.eu/, accessed May 2016), were assigned to the study sites. Weather data were adapted from the monthly summary observations map of NOAA which compiles worldwide weather data (http://gis.ncdc.noaa.gov/maps/, accessed August 2016) by selecting the nearest weather station for each study site. After soil texture analysis, soil types were defined using the World Reference Base for Soil Resources (WRB) (IUSS Working Group, 2006).

Functional group classification

Ecosystem functions, such as dung decomposition and secondary seed dispersal, are most likely affected by the amount of dung taken and the direction of dung transport. Therefore, dung beetle species were assigned to three functional groups according to their dung processing behaviour: dwellers, tunnelers, and rollers (Doube, 1990). We follow the definition of these functional groups given by Cambefort and Hanski (1991). Tunnelling species include all species in the Geotrupidae, Scarabaeinae species (genera: *Caccobius*, *Copris*, *Euoniticellus*, *Euonthophagus*, *Onitis*, *Onthophagus*) and some Aphodiinae species (*Colobopterus erraticus* in particular, see Rojewski (1983) and Vitner (1998)) while rollers are the remaining Scarabaeinae

species (genera: *Gymnopleurus*, *Scarabaeus*, *Sisyphus*) and dwellers comprise almost all Aphodiinae species.

Furthermore, as the amount of dung being transported is strongly correlated with beetle size (Horgan, 2001), tunnelers and rollers were further subdivided into size classes, as either small or large, based on their ability to move through a 1 cm² mesh (square mesh with side lengths of 1 cm, as in e.g., Slade et al. (2007)). Dwellers are generally small species and were therefore not classified according to size. As other soil macro-invertebrates, such as earthworms and isopods, are often major dung feeders or decomposers (see e.g., Gittings et al. (1994), Gittings and Gillller (1999)) with a similar body width and dung removal strategy as small tunnelers, we defined a sixth functional group composed of other soil macro-invertebrates.

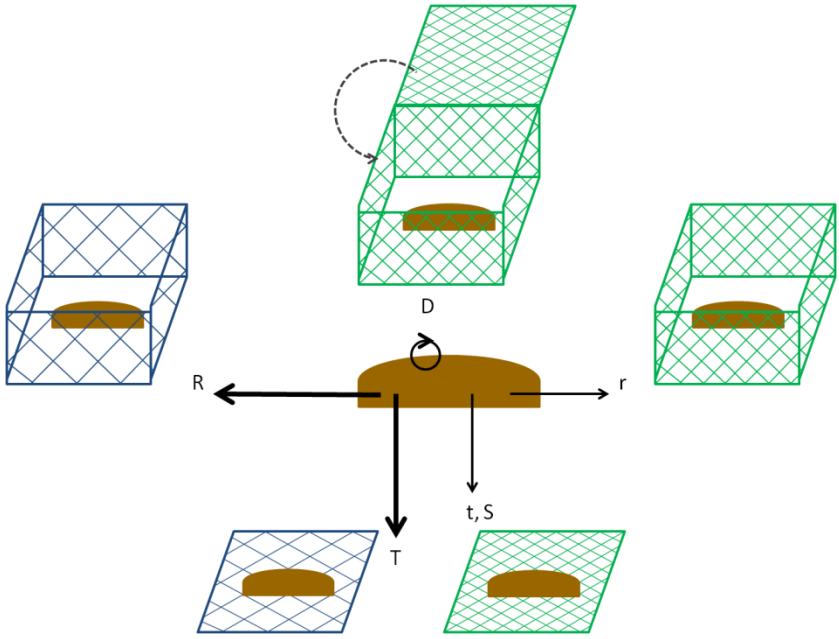

Figure 8.1 - Direction of dung transport by functional groups (with D= dwellers, R= large rollers, r= small rollers, T= large tunnelers, t= small tunnelers and S= other soil macro-invertebrates) and the combinations of walls, ground screens and ceilings (in either small sized mesh (1 mm², green) or coarse sized mesh (1 cm², blue)) needed to prevent dung removal by each group.

Experimental design

At each study site, an experimental area of ca. 300 m² was fenced in order to prevent the interference of large herbivores with the experiment, while the dung fauna associated with these large herbivores could enter the experimental zone without restrictions. Before starting the experiments, the vegetation was cut to an average height of 3 cm in order to provide a levelled surface for the establishment of experimental plots. Eleven exclosure types were designed by combining different kinds of ground screens, vertical walls, and/or ceilings in order to prevent the dung removing activity of different combinations of functional groups (Figure 8.1 and Table 8.1). Ground screens were used to prevent dung being removed by tunnelers, while walls prevented the dung removing activity of rollers. By combining walls and ceilings, the activity of all dung beetle functional groups was prevented. Ground screens, walls and ceilings were made of plastic mesh and the mesh size determined the size class of the beetles being able to remove dung from the experimental unit. We used mesh sizes of 1 cm² (square mesh with side lengths of 1 cm) to include small beetles but to exclude large beetles and 1 mm² (square mesh with side lengths of 1 mm) to exclude all beetle activity. In treatments lacking ground screens or walls, tunnelers and rollers of all size classes were able to translocate dung. As dung beetles most often reach their feeding source by flying (Larsen and Forsyth, 2005), all treatments without ceilings allowed the entrance of any dung beetle functional group, but the removal of dung by tunnelers and rollers was prevented by the use of ground screens and walls. Dwellers, however, do not relocate dung in the same way, and dung removal by this group could only be prevented by excluding all dung beetle activity in a combination of walls and a ceiling in fine mesh material (treatments S and control). In treatments using a coarse-meshed ground screen or no ground screen, dung removing activities of soil fauna other than dung beetles and able to move through a 1 mm² mesh were included. Therefore, soil macro-invertebrates were included as a sixth functional group and its activity was measured in treatment S (Table 8.1). Each experimental unit had a square ground surface of 40 cm by 40 cm and walls were 15 cm high. Cattle dung was used as a reference dung type at all study sites, and at most study sites the experiment was replicated using other dung types (including horse, sheep, red deer or goat dung, see Appendix, Table A 8.3 for a complete list).

Table 8.1 - Treatments used in the dung and seed removal experiments. Materials used for ground screens, walls and ceilings: F= fine mesh (1 mm²), C= coarse mesh (1 cm²) and N= not present. Inclusion (+) and exclusion (-) of dung removal by different functional groups: dwellers (D), large and small tunnelers (resp. T and t), large and small rollers (resp. R and r), and other soil macro-invertebrates (S). Exclosure types marked with* were not built at study sites where no rolling species occur (Bavarian Forest NP, Castillo de Viñuelas, Le Chesnoy, Lyngheisenteret Lygra, Moorhouse NP, Steinbühl, Tähtvere parish, Vácrátót and Wytham woods in all experimental runs, and in Bugac, Bayreuth and Kalmthout during the experiments in 2015). In the control treatment dung degradation was measured in the absence of invertebrates unable to move through 1 mm² mesh.

		construction material			**6 functional groups**					
	treatment	**ground**	**wall**	**ceiling**	**dwellers (D)**	**large tunnelers (T)**	**small tunnelers (t)**	**large rollers (R)**	**small rollers (r)**	**soil macro-invertebrates (S)**
1	DTtRrS	N	N	N	+	+	+	+	+	+
2	DRr	F	N	N	+	-	-	+	+	-
3	DtRrS	C	N	N	+	-	+	+	+	+
4	DTtS*	N	F	N	+	+	+	-	-	+
5	DTtrS*	N	C	N	+	+	+	-	+	+
6	D*	F	F	N	+	-	-	-	-	-
7	DtrS*	C	C	N	+	-	+	-	+	+
8	Dr*	F	C	N	+	-	-	-	+	-
9	DtS*	C	F	N	+	-	+	-	-	+
10	S	N	F	F	-	-	-	-	-	+
11	Control	F	F	F	-	-	-	-	-	-

In study areas with no previous records of rolling species, treatments focussing specifically on rollers were discarded (Table 8.1). Each experimental unit was replicated six times for each dung type and units using the same dung type were grouped in blocks. Within each block, experimental units were set up in a fully randomized design. Individual experimental units were 60 cm apart, while different blocks were at least 2 m apart (see Appendix, Figure A 8.3).

Experimental plots excluding specific dung beetle functional groups at the Zwin, Belgium.

Dung removal

Dung was collected from animals that had not been treated with anthelmintics in the preceding 6 weeks in order to avoid interference of drug residues with dung beetle presence (Holter et al., 1993; Wardhaugh and Mahon, 1991). Once collected, dung was homogenized and frozen at -20 °C for at least two days in order to kill any biota able to consume large amounts of dung (as in e.g., Slade et al. (2007)). In some cases, dung was collected immediately after defecation by the animals in a stable and freezing was not required. Standardized amounts of dung (300 g

(experiments in 2014) or 500g (experiments in 2015) of cattle dung and 100 g of all other dung types) were put in the centre of each experimental plot and left on site for one month, when the remaining dung was collected and quantified.

At the start of the experiment, fresh subsamples of each dung batch were taken and weighed for use as a reference sample. Subsequently, reference samples were oven-dried and the dry weight was measured. At the end of the experiment, the remaining dung in the experimental units was collected, oven-dried and the dry mass was measured again. In the seed dispersal experiments, the amount of seeds left in the dung samples was counted.

Dung removal ratio was calculated as:

$$\mathrm{M_{removed}} = \frac{\mathrm{M_{initial}} - \mathrm{M_{final}}}{\mathrm{M_{initial}}}$$

where $M_{initial}$ is the average dry mass of the reference samples and M_{final} is the dry mass of the remaining dung at the end of the experiment.

Secondary seed dispersal

In the experiments performed in 2014, secondary seed dispersal was measured by adding seeds of *Alopecurus myosuroides*, *Galium aparine* and *Poa pratensis* to the dung samples. These species were selected for their differing shape and size, which is elongated and large (6 mm), spherical and medium-sized (3 mm) and elongated and small (1 mm) for respectively *A. myosuroides*, *G. aparine* and *P. pratensis*. Prior to the experiment, seeds were sterilized by dry heating at 80 °C during 7 days. The effectiveness of this treatment was confirmed as no seedlings appeared during a 60 day germination trial on 1 % agar under lab conditions (12 h day: 12h night regime with fluctuating temperatures between 14 °C and 30 °C). In order to increase the visibility of seeds in the dung samples, each seed species was spray-painted in distinct fluorescent colours. Ten seeds of each species were mixed with the homogenized dung portions at the start of the experiment. At the end of the experiment, the dried dung was crumbled and the remaining seeds were counted using visual detection and UV lights.

Seed dispersal ratio was expressed as:

$$S_{\text{dispersed}} = \frac{S_{\text{initial}} - S_{\text{final}}}{S_{\text{initial}}}$$

where $S_{initial}$ is the number of seeds put in the dung samples and S_{final} is the number of seeds retrieved from the samples at the end of the experiment.

Cattle dung with seeds of *Poa pratensis* (class small, blue), *Galium aparine* (class medium, yellow) and *Alopecurus myosuroides* (class large, pink). ©Carsten Eichberg

Dung beetle sampling

During the experiments, the dung beetle community was sampled in each study area. In the first year of the experiment, two types of pitfall traps were used in order to achieve a complete view of dung beetle diversity and abundance. The first trap type consisted of one large container (1 l) with a 11 cm wide opening at the top, and covered with hexagonal chicken wire (with a mesh diameter of 25 mm) and baited with dung packed in a nylon bag put on top of the chicken wire (as in Larsen and Forsyth (2005)). The second trap type consisted of five smaller containers (0.2 l) with a 7 cm wide

opening at the top, and surrounding a central dung pile (as in D'hondt et al (2008)). As the dung beetle species richness did not differ between trap types in the first year, sampling effort was lowered in the 2015 experiments by using the 1 l pitfall traps baited with 500 g of unwrapped dung (see Appendix, Figure A 8.5 for details). In all trap types containers were dug into the soil with the upper rim levelled with the soil surface. A saturated salt-water solution (ca. 365 g/l NaCl with some drops of unscented detergent) was used as a fixation fluid. Both trap types were set up randomly between the experimental units with six replicates per dung type (see Appendix, Figure A 8.4). Traps were put in operation one week after the start of the dung removal and seed dispersal experiments in order to avoid interference with the initial phase of the experiment. Traps were emptied weekly and sampling stopped with the end of the experiment after one month. All sampled Scarabaeoidea dung beetles species were identified to species level and assigned to one of the five functional groups. Dung beetle assemblages were defined by species diversity (number of species), total abundance per species, Shannon-Weaver diversity index (H') and evenness (J) (Hill, 1973) using the following formulae:

$$H' = -\sum_{i=1}^{S} p_i \ln (p_i)$$

$$J = \frac{H'}{\ln (S)}$$

where p_i is the proportion of individuals belonging to the i^{th} species in the dataset of interest and S is species richness. Furthermore, the number of missing species was estimated by Chao bias-corrected, first-order jack knife, second-order jack knife and bootstrap methods using the *specpool* function in the R package 'vegan' (Oksanen et al., 2016).

Statistical analysis

We applied non-metric multidimensional scaling (NMDS) ordination in order to examine whether dung beetle assemblage composition differed between study sites and regions. NMDS analyses were performed using the *metaMDS* function in the R package 'vegan, version 2.3-5' (Oksanen et al., 2016) which

uses a Bray-Curtis dissimilarity matrix of the species abundance data as a default. Weather and geographical data were fitted to the NMDS using the *envfit* function and their goodness of fit was assessed with 1000 permutation tests. Vegan's function *specaccum* with 100 random permutations was used to calculate species accumulation curves as a measure of sampling completeness in each study site. The relative abundance of each functional group per trap was analysed with a nested ANOVA with dung type, trap type, climate zone and biogeographic province as main factors, with study site nested within climate zone and biogeographic province. In case significant differences were found, Tukey posthoc tests were applied.

Hierarchical partitioning, which is a multiple regression technique that jointly considers all possible models in order to identify the most likely causal factors (Chevan and Sutherland, 1991; Mac Nally, 2000), was applied to estimate the independent effect of each functional group on dung removal. In this analysis all dung removal data were used of all tested dung types and in order to make the models as realistic as possible, the relative abundance of dung beetle functional groups was used as the explanatory variable. The abundance of each dung beetle functional group was estimated by the average number of beetles collected of each functional group found on each dung type for each study area and experimental period. By using an average value we corrected for sampling efforts and lost sampling containers. A quasi-binomial error structure was employed in the models and significance levels were achieved after running 1000 randomization tests. Both hierarchical partitioning and randomization tests were run with the R package 'hier.part ', version 1.0-4 (Walsh and Mac Nally, 2013). A generalized linear model (GLM) was used to model dung removal in each treatment with the relative abundance of each functional group and environmental parameters as explanatory variables, and a nested dung type: biogeographic region term in order to account for the fact that certain dung types were not used at each study site. The GLM was built with a binomial error structure and the relevance of each explanatory variable was assessed using Wald tests. The linear relation between dung removal and the number of functional groups able to enter each experimental unit was assessed using generalized mixed effects models (GLMMs, Zuur et al (2009)) with either biographical regions or climate zones as fixed effects and study sites and dung types as random effects.

Finally, the relationship between seed dispersal and dung removal was assessed using generalized mixed-effects models using the entire dataset, with dung types, seed sizes, and biogeographic region as fixed effects and nested biogeographic region: study site and dung type: study site terms as random effects as the tested dung types differed between study sites. A binomial error structure was used to incorporate the proportional seed dispersal and dung removal data in the model. The *glmer* function in the 'lme4' version 1.1-12 package (Bates et al., 2014) was used for all GLMM analyses. We used a backward selection approach to eliminate covariates based on Akaike's information criteria (AIC) (Bolker et al., 2009). The resulting model was tested for overdispersion and the significance of each explanatory variable was determined using Wald tests. Significant differences between dung types, seed sizes, and biogeographic regions were assessed using Tukey post-hoc tests with the function *glht* in the package 'multcomp' version 1.4-5 (Hothorn et al., 2008). All analyses were performed in R version 3.3.1. (R Core Team, 2016a).

Results

Dung beetle assemblage

In total 34994 specimens belonging to 94 dung beetle species were sampled and species were classified into four functional groups: dwellers, large and small tunnelers and small rollers. None of the samples contained large rollers (Appendix, Table A 8.4). Study sites differed both in species diversity and dung beetle abundance, with the highest number of species and specimens sampled at Bugac in the Pannonian region (Table 8.2 and Appendix, Figure A 8.5). Furthermore, the habitat specialisation differed along a geographic gradient with more habitat specialists being found at lower latitudes and in the eastern study sites (Figure 8.2). According to the species accumulation curves, sampling effort was sufficient in most biogeographic regions to make a realistic estimate of species richness, although individual sampling sites differed (Appendix, Figure A 8.5). Due to the low number of species and individuals sampled in Lygra, it was not possible to make an estimate for the West Eurasian taiga region. Dung

beetle assemblage composition differed clearly between biogeographic provinces and to a lesser extent between climate zones (Figure 8.3). Most of this variation could be attributed to the geographic location of the sampling sites (latitude, longitude and altitude, see Table 8.3), but climatologic measures during the sampling months, such as the mean and mean minimum temperature and the total monthly precipitation, also significantly affected the clustering of species in biogeographic and climate zones. In general horse and sheep dung attracted more dung beetle specimens compared to cattle dung, but this was not straightforward in all study sites. Functional group composition differed among biogeographic provinces and climate zones (Figure 8.4 and Appendix, Table A 8.5). Overall, dwellers were the most abundant and diverse group, but had a higher relative abundance in the northern regions (Atlantic, Boreal, British islands and West Eurasian taiga) compared to the study sites located at lower latitudes (Iberian highlands, Mediterranean sclerophyl, Middle European forest and to a lesser extent the Pannonian region) where dung beetle assemblages were mainly dominated by small tunnelers. Although rollers were found at two study sites, La Fage and Shahrekord (Appendix, Table A 8.4), their diversity and abundance were low. Furthermore, the relative abundance of functional groups differed between trap types as significantly more small tunnelers were found in the larger traps covered with dung (Appendix, Table A 8.5).

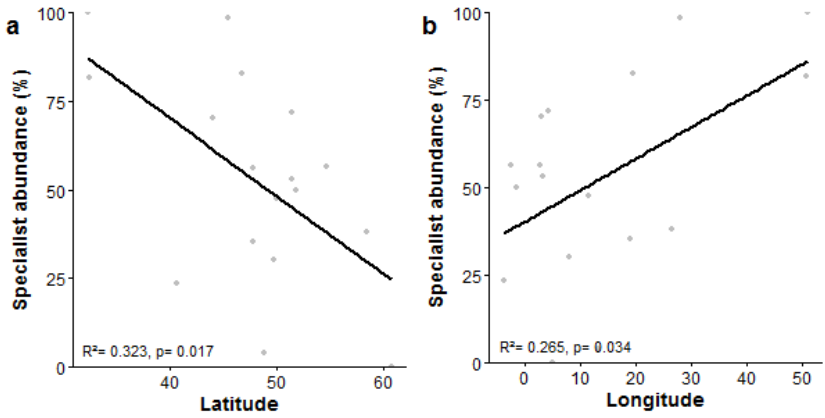

Figure 8.2 - Abundance of habitat specialists vs. latitude (a) and longitude (b). Regression results and *p*-values are plotted in the graphs.

Table 8.2 - Diversity indices for each Köppen-Geiger climate zone (Peel et al., 2007), biogeographic province (Udvardy, 1975) and study site (with mapping codes between brackets, see Appendix, Figure A 8.1 and Figure A 8.2). Climate zones are ordered according to latitude with Cfc= cold summer maritime temperate climate, Dfb= warm summer continental climate, Cfb= warm summer maritime temperate climate, Dfa= hot summer continental climate, Csb= dry and warm summer Mediterranean climate, and BWk= cold desert climate. Nt= number of baited pitfall traps, Ni= number of individuals, S= number of species, H'= Shannon-Weaver index, J= evenness, chao= Chao bias-corrected estimator (± 1 standard error), jack1= first-order jackknife (± 1 standard error), jack2= second-order jackknife, and boot= bootstrap estimator (± 1 standard error).

Climate zone	Biogeographic province	Study site	Country	N_t	N_i	S	H'	J	chao±SE	jack1±SE	jack2	boot±SE
Cfc				***36***	***3***	***1***	***0.00***	***-***	***1.0±0.0***	***1.0±0.0***	***1.0***	***1.0±0.0***
	West Eurasian taiga	Lygra (8)	Norway	36	3	1	0.00	-	1.0±0.0	1.0±0.0	1.0	1.0±0.0
Dfb				***209***	***12296***	***53***	***2.87***	***0.72***	***65.4±17.0***	***58.0±2.2***	***61.9***	***55.3±1.3***
	Boreonemoral	Tähtvere parish (14)	Estonia	36	136	9	1.83	0.83	9.5±1.3	10.0±1.0	10.0	9.5±0.7
	Central European highlands	*all C-E highlands sites*		*83*	*305*	*17*	*2.08*	*0.73*	*34.5±23.0*	*22.8±2.8*	*27.6*	*19.4±1.5*
		Bayreuth(10)	Germany	48	254	13	1.85	0.72	18.6±6.7	16.7±1.9	20.1	14.5±1.0
		Bavarian Forest NP (11)	Germany	35	51	8	1.11	0.53	11.8±5.1	11.8±3.1	13.7	9.7±1.7
	Pannonian	Bugac (13)	Hungary	90	11855	44	2.76	0.73	50.2±7.5	48.9±2.6	51.9	46.2±1.5
Cfb				***282***	***13598***	***39***	***2.21***	***0.6***	***46.15±8.2***	***45.0±3.0***	***49.0***	***41.0±1.7***
	British islands	*All British islands sites*		*48*	*609*	*9*	*0.94*	*0.43*	*9.2±0.7*	*10.0±1.0*	*9.1*	*9.7±0.8*
		Moor House NNR (2)	UK	36	569	4	0.74	0.54	4.0±0.0	4.0±0.0	3.1	4.1±0.4
		Wytham Woods (3)	UK	12	40	7	1.49	0.77	8.8±3.4	8. 8±1.3	9.7	7.8±0.8
	Atlantic	*All Atlantic sites*		*234*	*12989*	*37*	*2.11*	*0.58*	*48.2±13.1*	*43.0±3.0*	*47.9*	*38.9±1.7*
		Le Chesnoy (4)	France	48	4480	23	1.83	0.59	35.2±16.8	28.0±3.0	31.8	25.0±1.6
		The Zwin (6)	Belgium	72	885	15	1.78	0.66	16.0±1.8	17.0±1.4	17.0	16.0±0.8
		Kalmthout (7)	Belgium	84	7315	17	1.08	0.38	21.4±7.1	20.0±1.7	21.9	18.7±0.97
		Steinbühl (9)	Germany	30	309	12	1.62	0.65	17.7±6.9	15.8±1.9	19.5	13.6±1.0

Table 8.2 continued.

Climate zone	Biogeographic province	Study site	Country	N_t	N_i	S	H'	J	chao±SE	jack1±SE	jack2	boot±SE
Dfa				***90***	***4933***	***40***	***2.15***	***0.58***	***41.5±2.3***	***43.0±1.7***	***43.0***	***41.8±1.2***
	Middle European forest	Vácrátót (12)	Hungary	54	4760	36	2.11	0.59	37.5±2.2	38.9±1.7	39.0	37.7±1.1
	Pontian steppe	Braila Islands (15)	Romania	36	173	12	1.39	0.56	15.9±5.1	15.9±3.1	17.8	13.8±1.6
Csb				***16***	***3755***	***31***	***2.28***	***0.66***	***33.9±3.5***	***35.7±2.1***	***36.8***	***33.4±1.3***
	Mediterranean sclerophyl	INRA, La Fage (5)	France	10	3064	18	1.93	0.67	22.1±6.5	20.7±1.6	22.4	19.3±0.9
	Iberian highlands	Castillo de Viñuelas (1)	Spain	6	691	16	1.28	0.46	17.3±2.0	18.5±1.4	18.9	17.3±0.9
BWk				***120***	***409***	***18***	***1.87***	***0.65***	***21.2±3.5***	***23.4±3.2***	***24.6***	***20.7±2.2***
	Caucaso-Iranian highlands	Shahrekord (16)	Iran	84	371	18	1.88	0.65	22.2±4.2	24.0±3.5	26.0	20.9±2.2
		Tange Sayad (17)	Iran	36	38	3	0.96	0.87	3.0±0.0	3.0±0.0	2.8	3.0±0.2

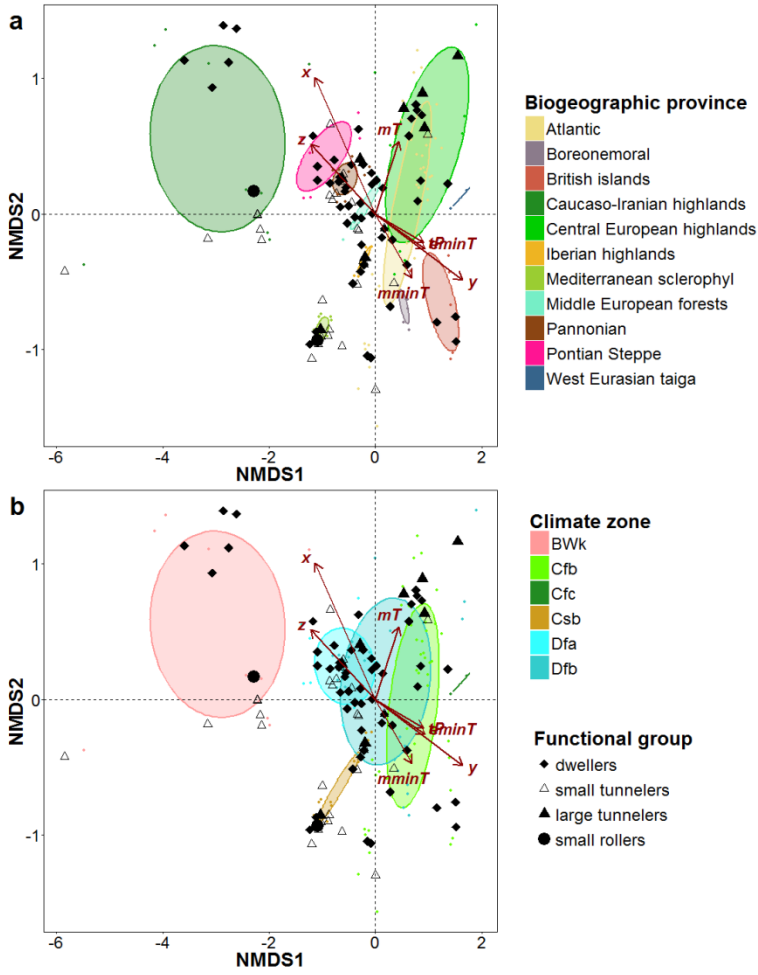

Figure 8.3 - Non-metric multidimensional scaling ordination of dung beetle assemblage of all study sites. Dung beetle species are plotted as functional groups and sampling locations (dots) are grouped by (a) biogeographic province sensu (Udvardy, 1975), and (b) by Köppen-Geiger climate zone (Peel et al., 2007) with Cfc= cold summer maritime temperate climate, Dfb= warm summer continental climate, Cfb= warm summer maritime temperate climate, Dfa= hot summer continental climate, Csb= dry and warm summer Mediterranean climate, and BWk= cold desert climate. Weather and location variables which explain a significant part of the observed variation are plotted as arrows, with x= longitude, y= latitude, z= altitude, mminT= mean minimum temperature, mT= mean temperature, eminT= lowest temperature, and eP= greatest observed precipitation. All climate variables are monthly summaries of the sampling month for each study site. The final stress value of the NMDS was 0.108. Full results of the fitted explanatory variables can be found in Table 8.3.

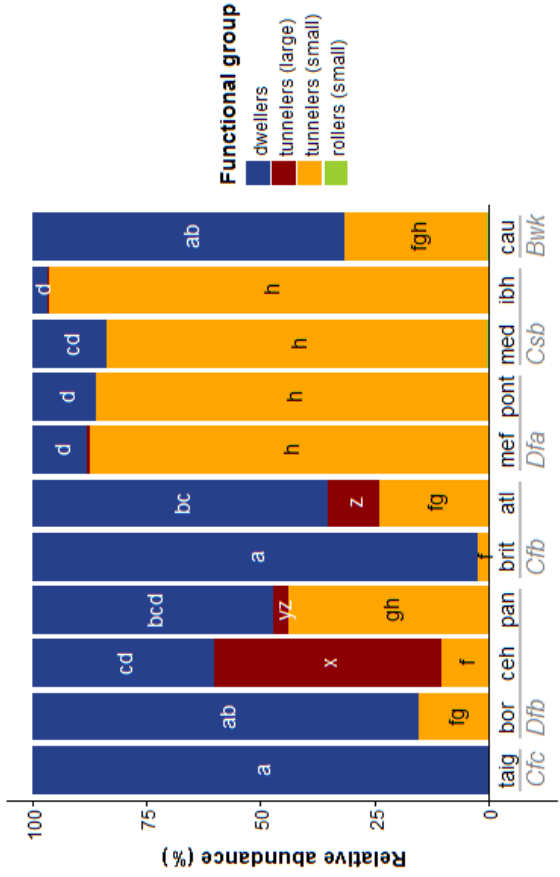

Figure 8.4 - Relative abundance of dung beetle functional groups by biogeographic province and Köppen-Geiger climate zone with atl= Atlantic, bor= Boreonemoral, brit= British islands, cau= Caucaso-Iranian highlands, ceh= Central European Highlands, ibh= Iberian highlands, med= Mediterranean sclerophyl, mef= Middle European forest, pan= Pannonian, pont= Pontian steppe and taig= West Eurasian taiga, Cfc= cold summer maritime temperate climate, Dfb= warm summer continental climate, Cfb= warm summer maritime temperate climate, Dfa= hot summer continental climate, Csb= dry and warm summer Mediterranean climate, and BWk= cold desert climate (Peel et al., 2007; Udvardy, 1975). Climate zones are ordered according to latitude. Small rollers represent 0.01 % and 0.06 % of the total number of sampled beetles in the Caucaso-Iranian and Mediterranean sclerophyl zones respectively and are invisible on the graph. Different letters indicate significant differences within functional groups after applying nested ANOVAs and Tukey posthoc tests. Full ANOVA results can be found in Table A 8.5.

Table 8.3 - R^2 and significance of weather and location variables fitted with the *envfit* function in R.

variable	R^2	*p*
longitude (x)	0.575	0.001
latitude (y)	0.730	0.001
altitude (z)	0.445	0.001
mean maximum temperature (mmaxT)	0.011	0.519
mean minimum temperature (mminT)	0.173	0.001
mean temperature (mT)	0.140	0.001
highest temperature (emaxT)	0.007	0.652
lowest temperature (eminT)	0.223	0.001
total precipitation (tP)	0.233	0.001
greatest observed precipitation (eP)	0.045	0.080
dung type	0.054	0.033
trap type	0.368	0.001

Dung removal

Each functional group was able to remove significant amounts of dung, although patterns differed between biogeographic and climate zones (Figure 8.5). Dwellers were the most important dung removers in the British Islands, Caucaso-Iranian, Iberian highlands and middle European forest sites, while small tunnelers outweighed the other functional groups in the Boreonemoral and Pannonian sites. Small rollers removed significant amounts of dung at the Caucaso-Iranian and Mediterranean sites. In most biogeographic provinces, the group of other soil macro-invertebrates significantly decomposed dung as well, especially at the study sites at higher latitudes (e.g., in the Cfc climate zone). Biogeographical regions differed in the amount of dung being decomposed with more dung removal in the Iberian and Mediterranean zones compared to the study sites in the Boreonemoral, British, Caucaso-Iranian, middle European and Pontian zones. In general, less deer dung was removed in comparison with all other dung types (Appendix, Figure A 8.7). Furthermore, dung decomposition was affected by weather conditions such as mean, minimum and maximum temperature and highest daily precipitation (Table 8.4 and Appendix, Figure A 8.7). A positive linear relation was found between dung removal and the number of functional groups included in a treatment (Wald χ^2= 16.625, p<0.001) (Figure 8.6). Biogeographic regions differed in the amount of dung being decomposed (Wald χ^2= 40.781, p<0.001) with more dung removal in

the Iberian and Mediterranean zones compared to the study sites in the Boreonemoral, British, Caucaso-Iranian, middle European and Pontian zones.

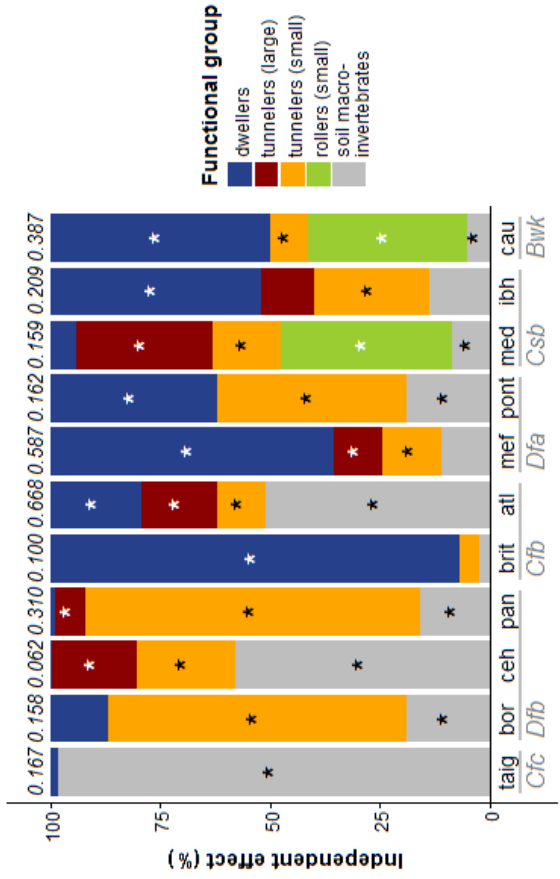

Figure 8.5 - Hierarchical partitioning results for cattle dung removal by dwellers, tunnelers, rollers and macro-invertebrates. The percentage of independent effect contributed by biogeographic province and Köppen-Geiger climate zone with atl= Atlantic, bor= Boreonemoral, brit= British islands, cau= Caucaso-Iranian highlands, ceh= Central European Highlands, ibh= Iberian highlands, med= Mediterranean sclerophyl, mef= Middle European forest, pan= Pannonian, pont= Pontian steppe and taig= West Eurasian taiga, Cfc= cold summer maritime temperate climate, Dfb= warm summer continental climate, Cfb= warm summer maritime temperate climate, Dfa= hot summer continental climate, Csb= dry and warm summer Mediterranean climate, and BWk= cold desert climate (Peel et al., 2007; Udvardy, 1975). Climate zones are ordered according to latitude. Asterisks indicate whether the contribution of a functional group is significant ($p<0.05$) in a biogeographic zone. The level of significance of R^2 of the independent contribution of each functional group was defined with randomization tests based on 1000 permutations. On top of each bar R^2 values of the total model is shown (see Appendix, Table A 8.6 for detailed results).

Table 8.4 - Results of the Wald test on the generalized linear model (GLM, R^2= 0.561) with dung removal as the response variable and mean abundance of functional groups, biogeographic regions and environmental parameters as explanatory variables. Predicted and observed dung removal for each of the significant climate variables are shown in Figure A 8.7.

variable	*df*	Wald χ^2	*p*
dwellers	1	3.447	0.063
large tunnelers	1	17.914	<0.001
small tunnelers	1	3.900	0.048
small rollers	1	13.738	<0.001
soil macro-invertebrates	1	210.855	<0.001
biogeographic region	10	322.231	<0.001
dung type	4	65.171	<0.001
mean maximum temperature (mmaxT)	1	2.341	0.126
mean minimum temperature (mminT)	1	0.267	0.605
mean temperature (mT)	1	0.625	0.429
maximum temperature (emaxT)	1	6.519	0.011
lowest temperature (eminT)	1	0.001	0.993
total precipitation (tP)	1	2.003	0.157
greatest observed precipitation (eP)	1	5.524	0.019

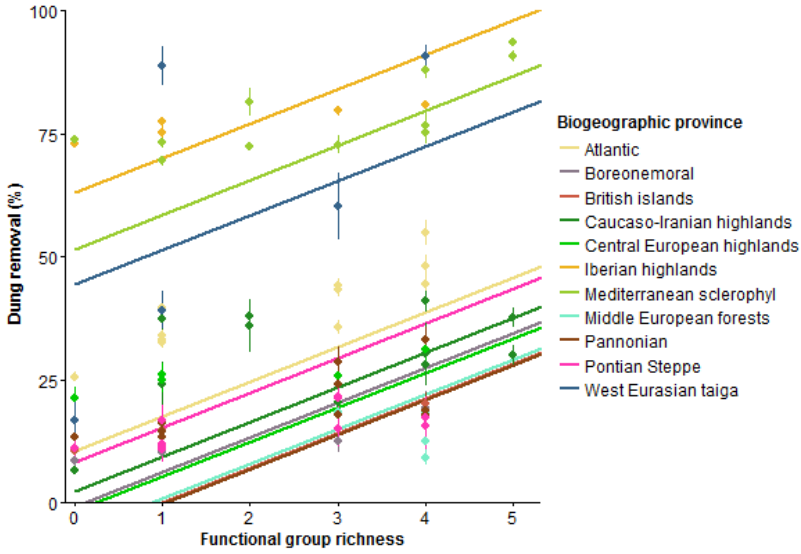

Figure 8.6 - Dung removal (%) in relation to functional group richness (no of functional groups) in each biogeographic province. Each dot represents the average percentage of dung removal in 1 treatment. Error flags represent 1 SE.

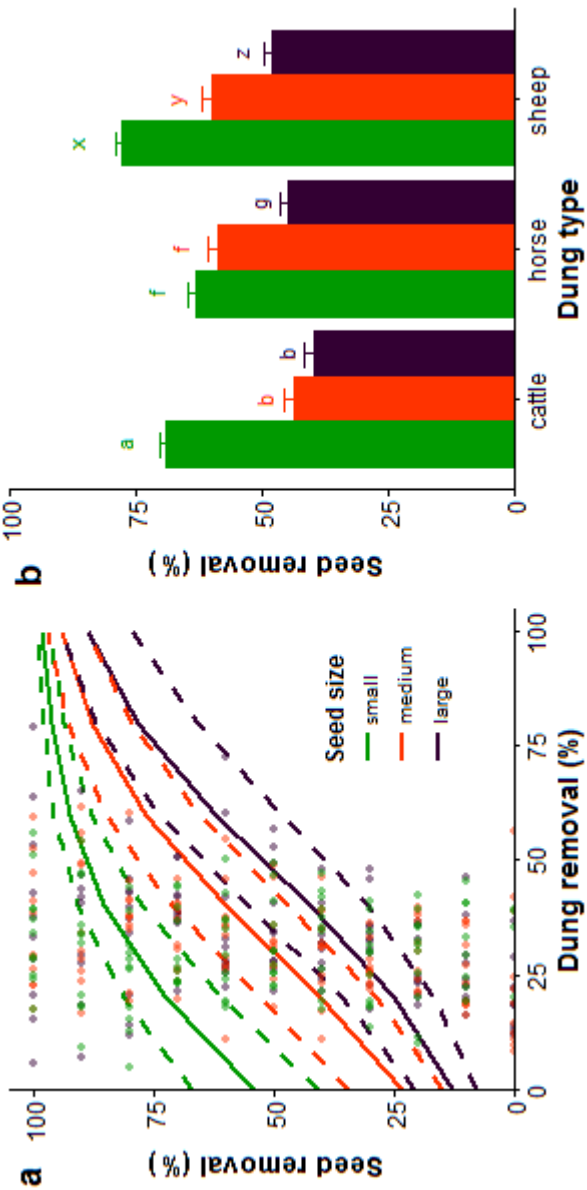

Figure 8.7 - (a) Predicted and measured seed removal vs. dung removal for each seed size class. Measured seed removal is plotted as dots. Predicted seed removal was obtained from the generalized linear model shown in Table 8.5 and is plotted as solid lines with the upper and lower confidence limits (dashed lines) and (b) seed removal by dung types and seed size classes. Error bars indicate standard errors. Different letters indicate differences between seed sizes within each dung type.

Secondary seed dispersal

A positive relation between secondary seed dispersal and dung removal was found (Figure 8.7 and Table 8.5). Furthermore, seed removal was affected by seed size as more small seeds were dispersed compared to medium (z= 10.089, p<0.001) and large-sized seeds (z= 10.935, p<0.001). No differences were found between dung types and biogeographic regions although a significant dung type: seed size interaction effect was found with more small-sized seeds being dispersed in sheep dung compared to horse and cattle dung, and more medium-sized seeds being dispersed in horse and sheep dung (Figure 8.7).

Table 8.5 - Wald test results on the generalized linear mixed-effects model (GLMM, R^2= 0.415) with seed dispersal as the response variable and dung removal, dung types, seed sizes and biogeographic region as fixed effects and nested dung type: study site and biogeographic region: study site as random terms. Predicted and observed seed removal vs. dung removal and for each of the dung type: seed size combinations are shown in Figure 8.7.

variable	*df*	Wald χ^2	*p*
dung removal	1	116.323	<0.001
dung type	2	4.649	0.098
seed size	2	246.618	<0.001
dung type x seed size	4	47.882	<0.001
biogeographic region	4	3.778	0.437

Discussion

Dung beetle assemblage

In the northernmost regions, dung beetle assemblages are dominated by dwellers while moving to the lower latitudes, tunnelers become more prominent. This corresponds well with the known biogeographic shift in dominance of dweller dominated assemblages at higher latitudes towards a dominance of tunnelers and rollers in southern regions (Hanski and Cambefort, 1991b; Verdú and Lobo, 2008). Remarkably, however, roller species remain scarce within the overall study area. Apart from the study

sites in La Fage, southern France and Shahrekord, Iran, no rollers have been sampled and even in these study sites their share in the overall dung beetle community was very small. Furthermore, the distribution of large tunneler species of the Geotrupidae family was mainly limited to the sites in the Atlantic, Central European highlands, and Pannonian region. Although the sampling efficiency was sufficient and at most sites species diversity fell within the range of expectations (see Hortal et al. (2011)), it is still possible that our sampled dung beetle assemblages were incomplete at the time of sampling related with stochastic variations in weather conditions, phenological population peaks, seasonality or the excess supply of resources in close by areas. In recent decades, dung beetle diversity has been under great pressure due to e.g., changes in land use (Barbero et al., 1999; Carpaneto et al., 2007; Lobo, 2001), the abandonment of cattle farming and resource scarcity as a consequence (Barbero et al., 1999), the use of veterinary medicines that interfere with the developmental stages of arthropods (Errouissi and Lumaret, 2010; Jochmann and Blanckenhorn, 2016; Lumaret et al., 2012; Ridsdill-Smith, 1993; Römbke et al., 2010), climate change (Dortel et al., 2013; Graham and Grimm, 1990; Lobo et al., 2007), the intensification of agricultural practices and policy promoting stabled cattle breeding with resource scarcity as a result (Hutton and Giller, 2003) and the scarcity of wildlife dung (Feer and Boissier, 2015). Especially the abundance and diversity of roller species, particularly large roller species, have declined in southern Europe since the 1950s, which is attributed to changes in land use and increasing urban development (Carpaneto et al., 2007; Lobo, 2001).

While dung beetle communities clearly differ between the northern (West Eurasian taiga, British islands, Boreonemoral sites) and the southern regions (Iberian highlands, Middle European forests, Mediterranean sclerophyl, Pannonian and Caucaso-Iranian sites), a further differentiation was found according to longitude with differing species composition between the eastern, more continental sites (Caucaso-Iranian, Pannonian and Middle European forests) and the western, more maritime study areas. Among biogeographic regions there is generally a clear difference in species composition with the exception of the Atlantic and Central European highland sites. This overlap could be caused by the fact that both regions cover a large area, both on a north-south and east-west axis, and contained more sampling sites than the other regions. Furthermore, species

assemblages are clearly affected by weather conditions. Dung beetle species assemblages were mostly affected by minimum and mean temperatures during the sampling period while maximum temperatures were not a discriminating factor. Davis et al. (2002) state that dung beetles most probably originated in warm tropical climates during the Mesozoic; they suggest them to be a warmth adapted insect group whose distribution is constrained by low temperature and the current and last-glacial location of the 0°C isotherm (Hortal et al., 2011). Furthermore, recent research in temperate and Mediterranean Europe pointed out that the current dung beetle distribution is largely defined by minimum temperatures (Lobo et al., 2002; Menéndez and Gutiérrez, 2004), and will likely change due to climate change resulting in more diverse dung beetle assemblages in the north and lowered diversity in the south (Dortel et al., 2013). Although the present study does not include dung beetle assemblage shifts in time, the north-south and east-west assemblage composition differences suggest the same prospects of future dung beetle assemblages.

Dung removal

Dwellers, being the most abundant functional group in the northern regions, did not contribute very much to dung removal in these regions. Dwellers in the Iberian highlands and Middle European forests on the other hand, removed a significant amount of dung although the functional group was underrepresented in the sampled dung beetle assemblage. Possibly, larval development and dung consumption by dweller larvae is faster at these sites compared to the more northern sites, possibly due to the higher summer temperatures (Lactin et al., 1995; Stevenson and Dindal, 1985). Furthermore, in the interpretation of the dung removal results for dweller species, the experimental set-up needs to be reconsidered. As the experiment was designed as an open system that allowed the interaction of the local dung fauna with experimental dung pats rather than using closed systems with a pre-defined number of species and densities, we could not prevent the dung manipulating action of other coprophilous fauna. For example, the treatment that excluded the dung removing action of rollers and tunnelers by using a fine grid ground screen and walls did prevent tunnelling or rolling dung balls, but tunnelers and rollers could still enter the treatment plot and feed on dung. Furthermore, except for the covered

treatment plots excluding all dung beetle activity, none of the treatment plots prevented oviposition by dung flies. As the larvae of dung-breeding flies play an important role in the dung ecosystem and have an abundant and global distribution (Hanski and Cambefort, 1991b), the measured dung removal by dwellers might be partially to be attributed to dung flies. In terms of ecosystem functioning, dung removal of dwellers and their larvae, feeding of rollers and tunnelers, and dung-feeding of dung flies are comparable, as none of these groups transport dung and seeds in a horizontal or vertical direction.

Another dung manipulating group with similar functional traits as tunnelers are digging soil macro-invertebrates whose dung removing activity was measured in the covered treatment plots without ground screens. Many other invertebrate groups are often found in dung, but are rarely considered as true members of the dung community (Floate, 2011). Such species are most often found in the later stages of dung degradation and are rather casual visitors from adjacent habitats such as typical detritus feeders which are found in rich organic soils or decaying plant material, e.g., woodlice (Isopoda), millipedes (Diplopoda), earwigs (Dermaptera), springtails (Collembola), and earthworms (Oligochaeta). Especially at high and intermediate latitudes, significant amounts of dung are being removed by digging fauna other than dung beetles. This is notably the case for the site in Lygra, Norway: especially in the cool and wet climate of northern Europe, earthworms can fulfil an important role in dung decomposition (Gittings and Giller, 1999; Gittings et al., 1994). Interspecific interactions between earthworms and dung beetles may also impact dung removal rates. Holter (1979) found an accelerated aggregation of earthworms underneath dung pats when dwellers and their larvae were present, resulting in a higher dung decomposition rate. On the other hand, in a laboratory experiment with different combinations of species groups, O'Hea et al. (2010) found a negative effect of beetle and earthworm interactions on dung decomposition while the combination of dung beetles and flies did not affect removal rates.

In our experiment, we found a general trend of an increasing dung removal rate with increasing functional group richness, which opposes the findings of O'Hea et al. (2010) of interspecific competition between dung beetles and earthworms and confirms those of Holter (1983) that dung becomes more attractive to earthworms when dung beetles are present. According to

Rosenlew and Roslin (2008), more dung is removed by large tunnelers compared to earthworms when both groups were present. In our study, however, soil macro-invertebrates exceeded large tunnelers in dung removal in the Atlantic and Central European highlands sites although generally high numbers of large tunnelers were sampled. In terms of ecosystem functions, both earthworms and tunnelers are similar as both species groups vertically transport dung and increase soil respiration, and water and carbon content (Hendriksen, 1997).

In the biogeographic provinces where small rollers were present, a high amount of dung was removed by this group, which contrasts with some research results from the tropics where tunnelers are considered the most important dung removers (Slade et al., 2007) and where larger beetles account for more dung removal (Braga et al., 2013). The high contribution of rolling species in the Mediterranean province also stresses the potential consequences of the on-going decline in the abundance and diversity of roller species in this zone of Europe for the ecosystem functions of dung removal and secondary seed dispersal (Carpaneto et al., 2007; Lobo, 2001).

Seed removal

The strong positive correlation between secondary seed dispersal and dung removal, independent from seed size, suggests that dung beetles did not distinguish between seed-containing dung and seed-free dung. This relation has been found earlier for relatively small seeds (e.g., 4 mm seeds in Andresen (2002a), 3.5 mm in Slade et al (2007)) although the number of buried seeds increases with dung pat size (Andresen and Levey, 2004) and dung beetle size (Andresen and Feer, 2005). The correlation might change to a negative relationship, if the proportion of seeds is higher (Shepherd and Chapman, 1998). Our results indicate that more small seeds were vertically dispersed compared to medium and large-sized seeds. However, we should interpret this result with caution as particularly the small seeds were poorly visible and difficult to retrieve. Furthermore, in contrast with most previously mentioned studies of secondary seed dispersal by dung beetles, we used real seeds instead of plastic proxies. Although the use of real seeds is more consistent with the actual processes of secondary seed dispersal, dung beetles might handle real seeds with a more variable morphology and

smell differently. Also, as we counted the remaining seeds, the removed seeds were either buried with dung particles by dung beetles or macro-invertebrates, or predated directly from the dung samples.

Secondary seed dispersal by dung beetles may have a direct impact on the reproduction success of plants (Nichols et al., 2008; Shepherd and Chapman, 1998). Distributing seeds from their original dropping site can help lowering the level of competition between seedlings (Andresen and Levey, 2004) and lower the impact of seed predators (Manzano et al., 2010). This is mainly achieved by rollers as they move dung away in a horizontal direction and bury dung shallowly. Therefore, they do not constrain seed germination. On the other hand, tunnelers and earthworms both make vertical shafts below the dung pat. Nevertheless, the fate of secondary dispersed seeds might differ between both groups as earthworms generally deposit consumed dung as casts in the soil in the upper 2 cm where most plant species should be able to germinate (Hendriksen, 1997), while tunnelers bury seeds at varying depths with some tunneler species digging up to 150 cm deep burrows (e.g., *Typhaeus typhoeus* see Brussaard (1985)). Once seeds are buried, two post-dispersal seed fates are possible: germination or death. One of the main determinants of whether seeds are germinable following secondary dispersal is the depth at which they are buried (Andresen and Feer, 2005), which differs between dung beetle species (D'hondt et al., 2008) and the specific germination requirements of the plant species (Bliss and Smith, 1985; Limón and Peco, 2016). For example, in a coastal grassland in Belgium, D'hondt et al (2008) found a reduced germination success of temperate grassland seeds due to the burying activity of large tunnelers. By contrast, another study, carried out in a tropical forest with all functional groups present, concluded that dung beetle activity generally resulted in a higher seedling establishment (Andresen and Levey, 2004). Furthermore, the positive interaction between functional group richness and dung removal rates stresses the need of functional group conservation in order to maintain ecosystem functions of dung removal and the hereto linked secondary seed dispersal.

Conclusions

Dung beetle assemblages clearly differed along a north-south and east-west gradient, with higher species richness and dung beetle abundance at lower latitudes. Northernmost sites were dominated by dwellers while a functional shift towards more tunnelers was found in the southern sites. Rollers were found in some regions but with very low abundance and species richness. The higher species richness and dung beetle abundance in the southern sites resulted in higher dung removal rates. Tunnelers and rollers were more effective dung removers than dwellers while other soil macro-invertebrates partially took over the dung removing activities of tunnelers in the dweller dominated northern sites. Furthermore, the positive interaction between functional group richness and dung removal rates stresses the need of functional group conservation in order to maintain ecosystem functions of dung removal and the herewith linked secondary seed dispersal. As the distribution of dung beetle functional groups is closely linked with the current minimum temperatures, an increase of the lowest temperatures might cause a northward migration of certain species. As a result, global change and the altered functional composition of dung beetle assemblages could have a profound effect on future dung removal and secondary seed dispersal patterns.

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Appendix

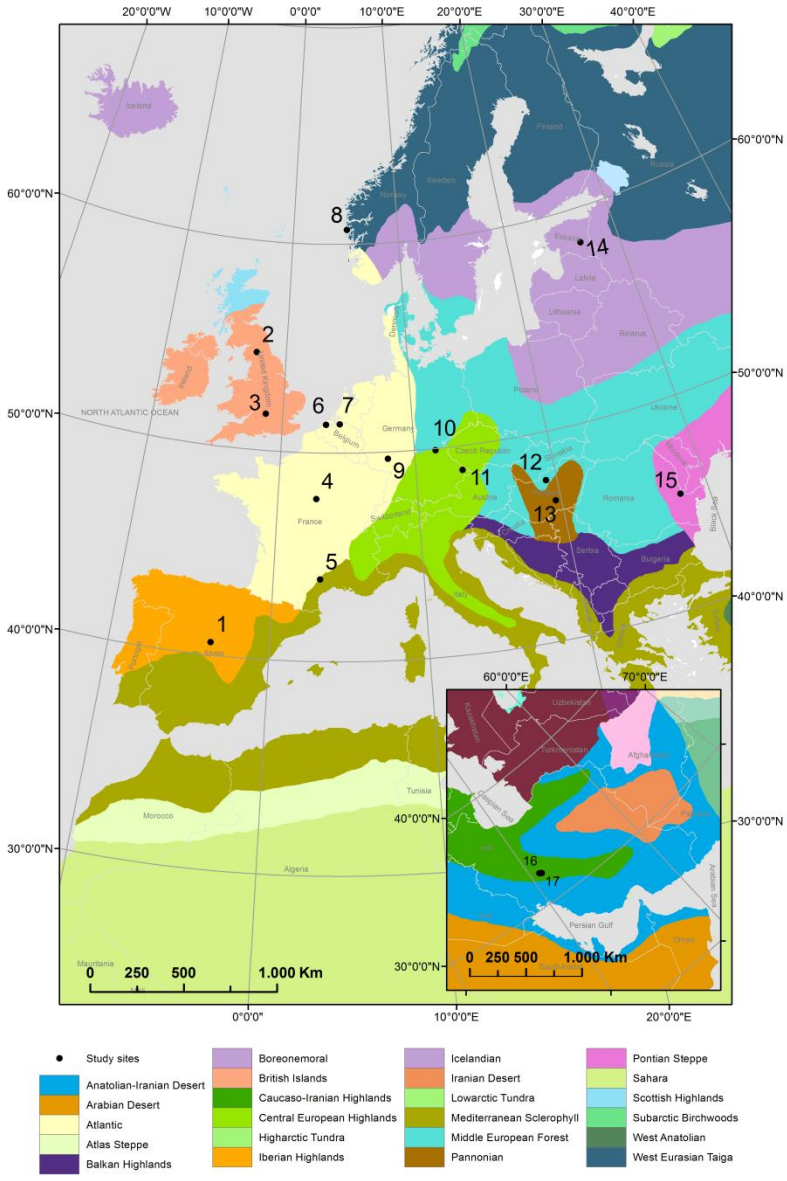

Figure A 8.1 - The study sites in Europe and Iran (inset) and their location in biogeographic provinces (Udvardy, 1975). The digital base map is adapted from FAO Geonetwork (2015).

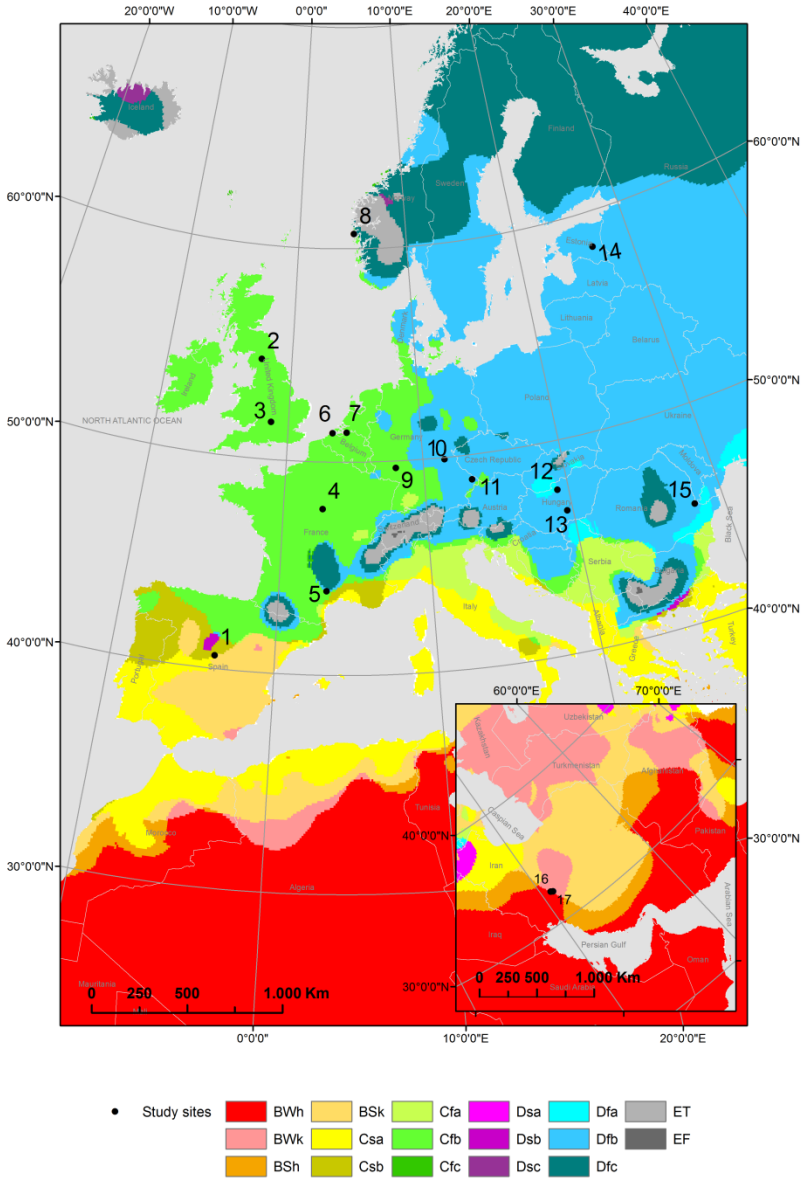

Figure A 8.2 - The study sites in Europe and Iran (inset) and their location within Köppen-Geiger climate zones. The digital base map is adapted from Peel et al. (2007).

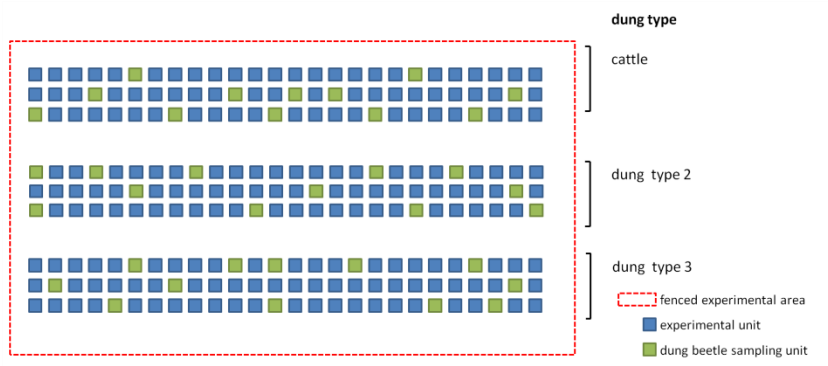

Figure A 8.3 - Experimental design with the random distribution of experimental units and sampling units grouped by dung types in an exclosure.

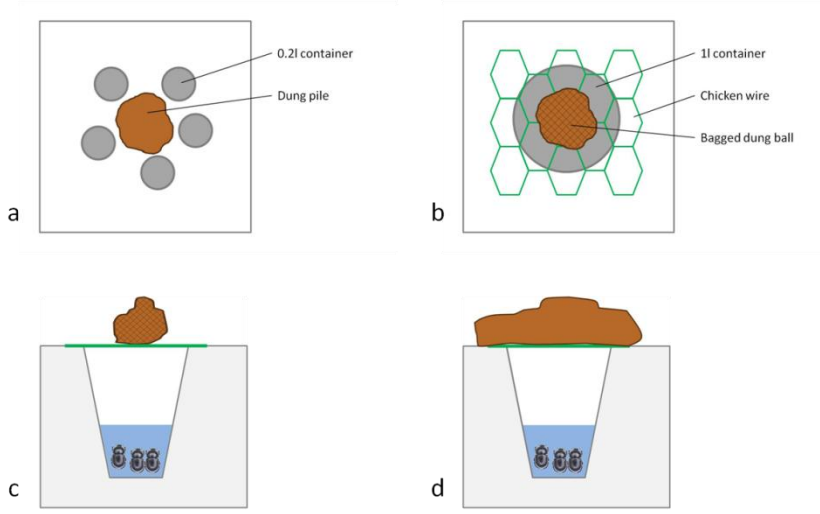

Figure A 8.4 - Schemes of the used trap types: (a) 5 small containers surrounding 1 central dung pat, (b-c) 1 large container covered by a ball of dung wrapped in nylon (b: top view, c: side view) and (d) 1 container topped with 1 large (unwrapped) dung pat (side view).

Table A 8.1 - Longitude, latitude and altitude of the study sites.

mapping code	country	name	latitude	longitude	altitude (m)
1	Spain	Castillo de Viñuelas	40° 36' 49'' N	3° 39' 50'' W	680
2	United Kingdom	Moor House National Nature Reserve	54° 39' 28'' N	02° 28' 05'' W	446
3	United Kingdom	Wytham Woods	51° 46' 11'' N	01° 19' 14'' W	80
4	France	Le Chesnoy	47° 47' 07'' N	02° 44' 55'' E	90
5	France	INRA, La Fage	43° 55' 31'' N	3° 6' 34'' E	780
6	Belgium	The Zwin	51° 21' 45'' N	03° 22' 02'' E	3
7	Belgium	Kalmthout	51° 23' 32'' N	04° 26' 05'' E	18
8	Norway	Lyngheisenteret, Lygra	60° 41' 14'' N	05° 07' 44'' E	20
9	Germany	Steinbühl	49° 40' 54'' N	08° 00' 02'' E	320
10	Germany	Bayreuth	49° 55' 02'' N	11° 35' 17'' E	355
11	Germany	Bavarian Forest National Park	48° 49' 58'' N	13° 23' 53'' E	1150
12	Hungary	Vácrátót	47° 42' 21'' N	19° 13' 47'' E	176
13	Hungary	Bugac	46° 39' 23'' N	19° 37' 10'' E	106
14	Estonia	Tähtvere parish	58° 22' 20'' N	26° 35' 01'' E	67
15	Romania	Braila Islands	45° 25' 08'' N	28° 02' 47'' E	2
16	Iran	Shahrekord	32° 21' 43'' N	50° 49' 52'' E	2055
17	Iran	Tange Sayad	32° 16' 42'' N	51° 01' 17'' E	2113

Table A 8.2 - Classification of the study sites in biogeographic provinces (Udvardy, 1975), Köppen-Geiger climate zones (Peel et al., 2007), habitat types and soil types.

Mapping code	study site	biogeographic province	Climate zone[1]	EUNIS habitat type[2]	soil type[3]
1	Castillo de Viñuelas	Iberian highlands	Csb	Mediterranean montane grassland	vertic luvisol
2	Moor House NP	British islands	Cfb	montane grassland	humic gleysol
3	Wytham Woods	British islands	Cfb	permanent mesotrophic pastures and aftermath-grazed meadows	eutric cambisol
4	Le Chesnoy	Atlantic	Cfb	grasslands	gleyic luvisol
5	INRA, La Fage	Mediterranean sclerophyl	Csb	Mediterranean montane grassland	eutric cambisol
6	The Zwin	Atlantic	Cfb	permanent mesotrophic pastures and aftermath-grazed meadows	calcaric fluvisol
7	Kalmthout	Atlantic	Cfb	temperate shrub heathland	humic podzol
8	Lygra	West Eurasian taiga	Cfc	grasslands	orthic podzol
9	Steinbühl	Atlantic	Cfb	permanent mesotrophic pastures on former arable land	dystric cambisol
10	Bayreuth	C-European highlands	Dfb	permanent mesotrophic pastures and aftermath-grazed meadows	stagnic gleysol
11	Bavarian Forest NP	C-European highlands	Dfb	permanent mesotrophic pastures and aftermath-grazed meadows	dystric cambisol
12	Vácrátót	Middle European forest	Dfa	grasslands	chromic cambisol
13	Bugac	Pannonian	Dfb	grasslands	calcaric regosol
14	Tähtvere parish	Boreonemoral	Dfb	permanent mesotrophic pastures and aftermath-grazed meadows	eutric podzoluvisol
15	Braila Islands	Pontian Steppe	Dfa	grasslands	calcaric fluvisol
16	Shahrekord	Caucaso-Iranian highlands	BWk	perennial calcareous grassland and basic steppes*	calcic xerosol
17	Tange Sayad	Caucaso-Iranian highlands	BWk	perennial calcareous grassland and basic steppes*	calcic xerosol

[1] with BWk: cold desert climate, Cfb: warm summer maritime temperate climate, Cfc: cold summer maritime temperate climate, Csb: dry and warm summer Mediterranean climate, Dfa: hot summer continental climate, Dfb: warm summer continental climate

[2] as defined in the European nature information system (EUNIS) by the European Environmental Agency (http://eunis.eea.europa.eu/)

[3] as defined in the World Reference Base for Soil Resources (WRB) (IUSS Working Group, 2006)

* Biogeographic region and EUNIS habitat type were identified using the definitions of the types by the European Environmental Agency in case study areas fall outside the borders of the pan-European classification.

Table A 8.3 - Timing of the experiments, dung types used in the experiments and grazer species nearby to the study areas.

Mapping code	name	timing experiments	used dung types	nearby grazer species
1	Castillo de Viñuelas	June 2016	cattle	cattle
2	Moor House National Preserve	mid June-mid July 2014, July 2015	cattle, sheep	sheep
3	Wytham Woods	mid June-mid July 2014	cattle	cattle, sheep
4	Le Chesnoy	June 2014*, June 2015	cattle, sheep, red deer	cattle, horse, sheep, red deer
5	INRA, La Fage	May 2015	sheep	sheep
6	The Zwin	Aug 2014, mid Sept-mid Oct 2014	cattle, horse, sheep	cattle, horse, sheep
7	Kalmthout	Aug 2014, mid Sept-mid Oct 2014, mid Sept-mid Oct 2015	cattle, horse, sheep	cattle, horse, sheep
8	Lyngheisenteret, Lygra	Aug 2014	cattle, horse, sheep	sheep
9	Steinbühl	June 2014, June 2015	cattle, horse	cattle, horses
10	Bayreuth	June 2014, June 2015	cattle, horse, sheep	cattle, sheep
11	Bavarian Forest National Park	Aug 2014, July 2015	cattle, horse	cattle, horse, deer
12	Vácrátót	June 2015, mid Sept-mid Oct 2015	cattle, horse, sheep	cattle, horse, sheep
13	Bugac	June 2014, June 2015, mid Sept-mid Oct 2015	cattle, horse, sheep	cattle, horse, sheep
14	Tähtvere parish	June 2014, mid June-mid July 2015	cattle, sheep	cattle, sheep
15	Braila Islands	July 2014	cattle, horse, sheep	cattle, horse, sheep
16	Shahrekord	Sept 2013, Oct 2013, mid Oct-mid Nov 2014, June 2015	cattle, sheep, goat	cattle, sheep
17	Tange Sayad	Aug 2013, mid Oct-mid Nov 2013, mid Oct-mid Nov 2014, June 2015	cattle, sheep, goat	cattle, sheep

* Experimental run limited to 14 days due to adverse meteorological conditions

Table A 8.4 - Dung beetle species and functional groups by study area (numbered columns) and abundance and species richness by functional group, summed for all experimental runs at each study site. See Tables A8.1-A8.3 for detailed information of study site locations and timing of the experiments. Functional groups (FG) are coded as follows: D= dwellers, r= small rollers, T= large tunnelers, and t= small tunnelers. Habitat specialisation (HS) is coded as follows: G= generalists, S= specialists, and *na*= no data available. Habitat specialisation data were adapted from Buse et al. (2015), Carpaneto et al. (2011), Frolov and Akhmetova (2013), Gharakloo (2010), IUCN (2016), Janssens (1960) and Jessop (1986).

species	FG	HS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Geotrupidae																			
Anoplotrupes stercorosus (Scriba, 1791)	T	G	0	0	0	0	0	0	0	0	0	1	36	0	0	0	0	0	0
Geotrupes mutator (Marsham, 1802)	T	S	0	0	0	0	0	0	0	0	0	0	0	0	81	0	0	0	0
Geotrupes spiniger (Marsham, 1802)	T	G	0	0	0	0	0	11	118	0	0	1	0	10	2	0	0	0	0
Geotrupes stercorarius (Linnaeus, 1758)	T	G	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Trypocopris vernalis (Linneus, 1758)	T	S	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0
Typhaeus typhoeus (Linneus, 1758)	T	S	0	0	0	0	0	0	483	0	0	0	0	0	0	0	0	0	0
Scarabaeidae																			
Aphodiinae																			
Acanthobodilus immundus (Creutzer, 1799)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	4	5	0	0	0	0
Acrossus depressus (Kugelann, 1792)	D	S	0	321	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acrossus luridus (Fabricius, 1775)	D	S	0	0	0	1	456	0	0	0	1	0	0	0	0	0	0	1	0
Acrossus rufipes (Linnaeus, 1758)	D	G	0	4	1	0	0	91	67	3	25	4	2	0	0	0	0	0	0
Agrilinus ater (De Geer, 1774)	D	G	0	242	13	70	0	1	0	0	0	15	0	0	0	38	0	0	0
Aphodius fimetarius (Linnaeus, 1758)	D	G	0	0	3	36	0	2	5	0	1	48	0	73	108	4	0	0	0
Aphodius foetidus (Herbst, 1783)	D	S	13	0	0	1	0	0	0	0	0	0	0	0	29	0	0	0	0
Aphodius lapponum (Gyllenhal, 1808)	D	*na*	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aphodius sp.	D	*na*	0	0	0	1	0	0	0	0	0	0	1	0	0	2	4	0	0
Biralus satellitius (Herbst, 1789)	D	S	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

Table A 8.4 continued.

species	FG	HS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Bodilus ictericus (Laicharting, 1781)	D	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Bodilus lugens (Creutzer, 1799)	D	S	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Bodilopsis rufa (Moll, 1782)	D	G	0	0	2	0	0	61	24	0	1	0	0	27	8	0	0	0	0
Bodilopsis sordida (Fabricius, 1775)	D	S	0	0	0	0	0	0	0	0	0	0	0	9	1257	5	0	0	0
Calamosternus granarius (Linnaeus, 1767)	D	G	0	0	0	1	0	0	0	0	0	0	1	0	43	0	0	35	0
Calamosternus hyxos (Petrovitz, 1962)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Chilothorax distinctus (Müller, 1776)	D	G	0	0	0	0	4	0	6	0	0	0	1	51	6	0	0	0	0
Chilothorax lineolatus (Illiger, 1803)	D	*na*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chilothorax melanostictus (Schmidt, 1840)	D	S	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	1	4
Chilothorax sp.	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	146	17
Colobopterus erraticus (Linnaeus, 1758)	t	S	111	0	4	29	4	0	2	0	52	97	0	14	0	19	1	0	0
Coprimorphus scrutator (Herbst, 1789)	D	S	5	0	0	0	0	0	0	0	1	0	0	10	64	0	0	0	0
Esymus merdarius (Fabricius, 1775)	D	S	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0
Esymus pusillus (Herbst, 1789)	D	S	0	0	0	216	3	0	0	0	36	24	2	37	1	27	0	0	0
Eudolus quadriguttatus (Herbst, 1783)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Euheptaulacus sus (Herbst, 1783)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	1569	0	0	0	0
Euorodalus paracoenosus (Balthasar & Hrubant, 1960)	D	G	0	0	0	0	0	0	0	0	0	0	0	90	431	0	0	0	0
Euorodalus tersus (Erichson, 1848)	D	*na*	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eupleurus subterraneus (Linnaeus, 1758)	D	S	0	0	0	0	0	0	0	0	0	0	0	2	3	0	5	0	0
Labarrus lividus (Olivier, 1789)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	264	0	0	0	0
Loraphodius suarius (Faldermann, 1832)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Melinopterus consputus (Creutzer, 1799)	D	S	0	0	0	0	4	1	0	0	0	0	0	1	47	0	0	0	0
Melinopterus prodromus (Brahm, 1790)	D	G	0	0	0	5	0	89	1667	0	0	1	0	14	0	0	0	1	0

Table A 8.4 continued.

species	FG	HS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Melinopterus reyi (Reitter, 1892)	D	*na*	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
Melinopterus sphacelatus (Panzer, 1798)	D	G	0	0	0	0	0	68	42	0	0	0	0	0	0	0	0	0	0
Nialus varians (Duftschmid, 1805)	D	S	0	0	16	1754	0	0	0	0	0	0	0	1	4	0	2	0	0
Nimbus contaminatus (Herbst, 1783)	D	S	0	0	0	0	0	435	4737	0	0	0	0	0	0	0	0	0	0
Nimbus obliteratus (Panzer, 1823)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Nobiellus inclusus (Reitter, 1892)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	17
Nobius serotinus (Panzer, 1799)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	70	0	0	0	0
Otophorus haemorrhoidalis (Linnaeus, 1758)	D	G	2	0	0	421	0	25	1	0	49	19	6	58	694	16	1	0	0
Oxyomus sylvestris (Scopoli, 1763)	D	*na*	0	0	0	1	0	0	0	0	0	5	0	0	70	1	0	0	0
Parammoecius gibbus (Germar, 1817)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Phalacronothus biguttatus (Germar, 1824)	D	S	0	0	0	0	0	0	0	0	0	0	0	44	16	0	0	0	0
Planolinus fasciatus (Olivier, 1789)	D	S	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Plagiogonus putridus (Geoffroy, 1785)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	22	1	0	0	0	0
Rhodaphodius foetens (Fabricius, 1787)	D	S	0	0	0	0	0	34	30	0	0	0	0	23	1371	0	0	0	0
Sigorus porcus (Fabricius, 1792)	D	S	0	0	0	0	0	0	0	0	0	0	0	101	206	0	0	0	0
Subrinus sturmi (Harold, 1870)	D	*na*	0	0	0	0	0	0	0	0	0	0	0	0	121	0	0	0	0
Subrinus vitellinus (Klug, 1845)	D	*na*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Teuchestes fossor (Linnaeus, 1758)	D	G	0	0	1	19	0	7	4	0	3	32	0	0	0	24	0	0	0
Trichonotulus scrofa (Fabricius, 1787)	D	S	0	0	0	14	10	0	0	0	0	0	0	0	458	0	0	0	0
Volinus sticticus (Panzer, 1798)	D	G	0	0	0	0	0	30	9	0	0	0	2	4	1	0	0	0	0
Scarabaeinae																			
Caccobius mundus (Ménétriès, 1838)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
Caccobius schreberi (Linnaeus, 1767)	t	S	7	0	0	0	0	0	0	0	0	0	0	514	440	0	21	0	0

Table A 8.4 continued.

species	FG	HS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Copris hispanus (Linnaeus, 1764)	T	*na*	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Copris lunaris (Linnaeus, 1758)	T	S	0	0	0	0	0	0	0	0	0	0	0	3	127	0	0	0	0
Euonthophagus amyntas (Olivier, 1789)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	53	0
Euoniticellus fulvus (Goeze, 1777)	t	S	15	0	0	13	1	0	0	0	0	0	0	168	664	0	27	0	0
Euonthophagus gibbosus (Scriba, 1790)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Euonthophagus mostafatsairii (Palestrini, Varola & Zunino, 1979)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
Euoniticellus pallipes (Fabricius, 1781)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	53	0	0	0	0
Gymnopleurus qurosh (Montreuil, 2011)	r	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Onitis belial (Fabricius, 1798)	t	*na*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Onthophagus amirus (Kabakov, 1982)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Onthophagus coenobita (Herbst, 1783)	t	G	0	0	0	77	0	2	2	0	3	0	0	138	1	0	0	0	0
Onthophagus dorsosignatus (D'Orbigny, 1898)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Onthophagus emarginatus (Mulsant & Godart, 1842)	t	*na*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Onthophagus fissicornis (Steven, 1809)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0
Onthophagus fracticornis (Preyssler, 1790)	t	G	0	0	0	0	180	0	0	0	0	0	0	2268	316	0	0	0	0
Onthophagus furcatus (Fabricius, 1781)	t	*na*	81	0	0	0	0	0	0	0	0	0	0	84	149	0	0	0	0
Onthophagus gibbulus (Pallas, 1781)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	220	0	0	0	0
Onthophagus grossepunctatus (Reitter, 1905)	t	*na*	0	0	0	0	46	0	0	0	0	0	0	2	0	0	0	0	0
Onthophagus illyricus (Scopoli, 1763)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	242	59	0	0	0	0
Onthophagus joannae (Goljan, 1953)	t	G	3	0	0	199	641	0	0	0	0	6	0	0	0	0	0	0	0
Onthophagus lemur (Fabricius, 1792)	t	S	0	0	0	0	717	0	0	0	0	0	0	0	0	0	0	0	0
Onthophagus medius (Kugelann, 1792)	t	S	0	0	0	7	375	0	0	0	3	0	0	0	0	0	0	0	0

Table A 8.4 continued.

species	FG	HS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Onthophagus nuchicornis (Linnaeus, 1758)	t	S	0	0	0	0	0	0	0	0	0	0	0	23	433	0	0	0	0
Onthophagus opacicollis (Reitter, 1892)	t	*na*	431	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Onthophagus ovatus (Linnaeus, 1767)	t	G	0	0	0	1045	0	0	0	0	134	1	0	232	0	0	1	0	0
Onthophagus pygargus (Motschulsky, 1845)	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	46	0
Onthophagus ruficapillus (Brullé, 1832)	t	*na*	0	0	0	0	519	0	0	0	0	0	0	111	246	0	0	7	0
Onthophagus similis (Scriba, 1790)	t	G	0	0	0	0	0	28	116	0	0	0	0	0	0	0	0	0	0
Onthophagus sp.	t	*na*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
Onthophagus taurus (Schreber, 1759)	t	S	5	0	0	487	0	0	0	0	0	0	0	353	2188	0	102	0	0
Onthophagus vacca (Linnaeus, 1767)	t	*na*	11	0	0	60	0	0	0	0	0	0	0	21	18	0	0	0	0
Onthophagus verticicornis (Laicharting, 1781)	t	G	0	0	0	18	84	0	0	0	0	0	0	2	0	0	0	0	0
Sisyphus schaefferi (Linnaeus, 1758)	r	S	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0
total number			**691**	**569**	**40**	**4480**	**3064**	**885**	**7315**	**3**	**309**	**254**	**51**	**4760**	**11855**	**136**	**173**	**371**	**38**
dwellers (D)			24	569	36	2545	478	844	6592	3	117	148	15	575	6858	117	16	240	38
small rollers (r)			0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	2	0
large tunnelers (T)			2	0	0	0	6	11	603	0	0	2	36	13	210	0	0	0	0
small tunnelers (t)			665	0	4	1935	2568	30	120	0	192	104	0	4172	4787	19	157	129	0
Species richness			**16**	**4**	**7**	**23**	**18**	**15**	**17**	**1**	**12**	**13**	**8**	**36**	**44**	**9**	**12**	**18**	**3**
dwellers (D)			6	4	6	14	6	12	11	1	8	8	7	20	29	8	6	8	3
small rollers (r)			0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
large tunnelers (T)			1	0	0	0	1	1	3	0	0	2	1	2	3	0	0	0	0
small tunnelers (t)			9	0	1	9	10	2	3	0	4	3	0	14	12	1	6	9	0

* *Aphodius fimetarius* s.l. should be regarded as a species complex of two distinct species, as genomic and morphologic differences exist between *Aphodius fimetarius* and *Aphodius pedellus* (Miraldo et al., 2014), or more recently *A. fimetarius* and *Aphodius cardinalis* (Fery and Rössner, 2015) have been described.

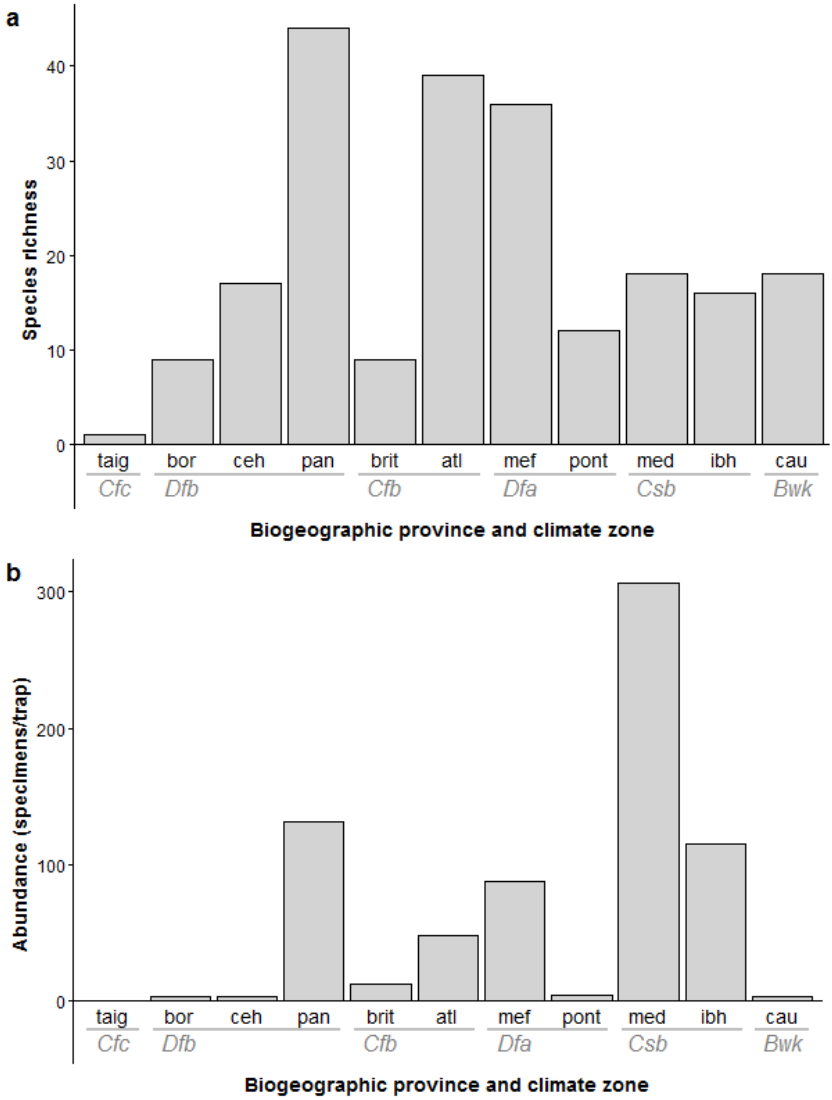

Figure A 8.5 - (a) Summed species richness by biogeographic province, and (b) dung beetle abundance by biogeographic province.

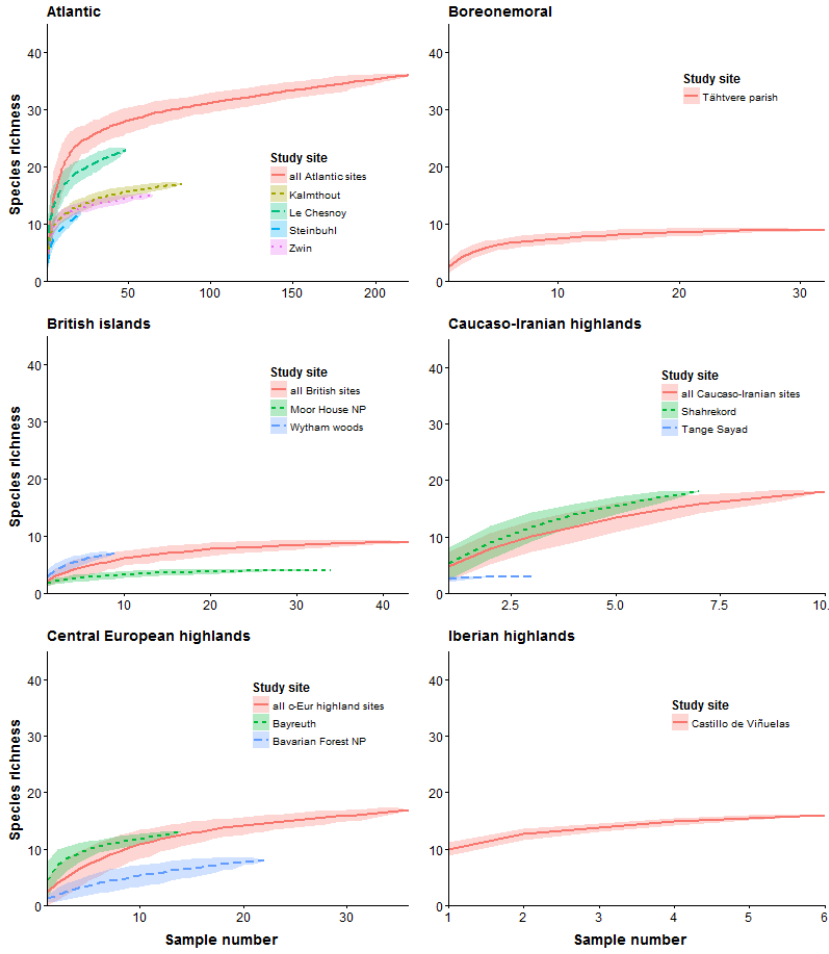

Figure A 8.6 - Randomized species accumulation curves using sample numbers as a measure of sampling effort accomplished for each study site (except Lygra) in each of the 11 regions studied.

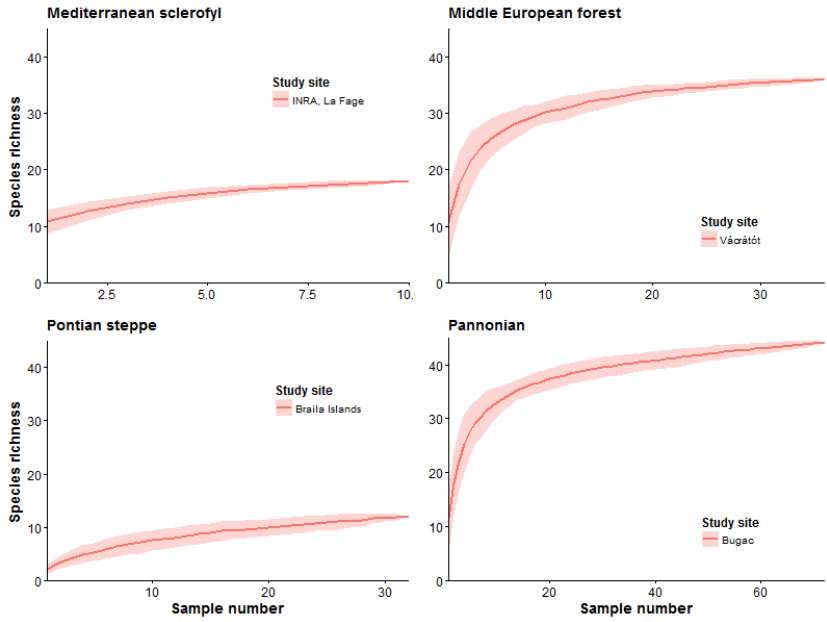

Figure A 8.6 continued.

Table A 8.5 - Results of the nested ANOVAs with the mean abundance of dung beetle functional groups as response variable and dung type, trap type, climate zone and biogeographic province as explanatory variables with study site nested within climate zone and biogeographic province.

functional group	**variable**	***df***	***F* value**	***p***
dwellers	dung type	4	3.752	0.008
	trap type	1	2.201	0.142
	climate zone	5	14.934	<0.001
	biogeographic province	5	5.072	<0.001
	climate zone: study site	6	7.967	<0.001
small tunnelers	dung type	4	1.866	0.125
	trap type	1	6.407	0.013
	climate zone	5	25.026	<0.001
	biogeographic province	5	6.760	<0.001
	climate zone: study site	6	11.766	<0.001
large tunnelers	dung type	4	4.280	0.003
	trap type	1	2.036	0.157
	climate zone	5	7.155	<0.001
	biogeographic province	5	19.355	<0.001
	climate zone: study site	6	22.750	<0.001
small rollers	dung type	4	0.323	0.862
	trap type	1	1.819	0.181
	climate zone	5	6.638	<0.001
	biogeographic province	5	2.656	0.028
	climate zone: study site	6	0.522	0.522

Table A 8.6 - R^2 values for the hierarchical partitioning analysis with dung removal as the response variable. Individual R^2 values for each functional group and the total R^2 for all groups are presented. Significant explanatory variables after applying randomization tests are marked with asterisks. See Figure 8.5 for the graphic presentation of the results.

	explanatory variable individual R^2					
response variable	**dwellers**	**tunnelers (large)**	**tunnelers (small)**	**rollers (small)**	**macro-invertebrates**	**total R^2**
biogeographic province						
Atlantic (atl)	0.044*	0.037*	0.006*		0.090*	0.167
Boreonemoral (bor)	0.000		0.111*		0.071*	0.158
British islands (brit)	0.060*		0.004		0.002	0.062
Caucaso-Iranian highlands (cau)	0.256*	0.000	0.067*	0.193*	0.043*	0.310
Central European highlands (ceh)	0.000	0.021*	0.025*		0.069*	0.100
Iberian highlands (ibh)	0.533*	0.271	0.488*		0.214*	0.668
Mediterranean sclerophyl (med)	0.074	0.317*	0.248*	0.288*	0.155	0.587
Middle European forest (mef)	0.133*	0.043*	0.023*		0.013*	0.162
Pannonian (pan)	0.006	0.032*	0.157*		0.064*	0.159
Pontian steppe (pont)	0.076*		0.107*		0.076*	0.209
West Eurasian taiga (taig)	0.014				0.386*	0.387
climate zone						
BWk	0.256*	0.000	0.067*	0.193*	0.043*	0.310
Cfb	0.033*	0.044*	0.023*		0.077*	0.136
Cfc	0.014				0.386*	0.387
Csb	0.078*	0.236*	0.241*	0.242*	0.143*	0.508
Dfa	0.096*	0.007	0.050*		0.036*	0.162
Dfb	0.007*	0.000	0.049*		0.014*	0.053

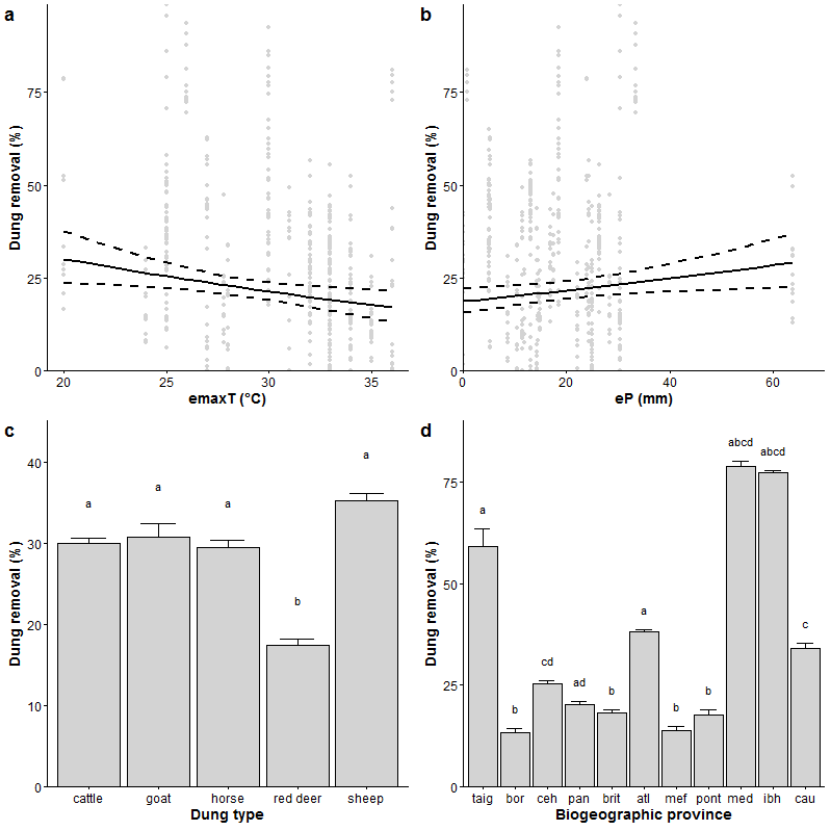

Figure A 8.7 - Predicted and measured dung removal vs. extreme maximum temperature (emaxT) (a) and extreme precipitation (eP) (b). Measured dung removal is plotted as grey dots. Predicted dung removal was obtained from the generalized linear model shown in Table 8.4 and is plotted as solid lines with the upper and lower confidence limits (dashed lines). Mean dung removal by dung types (c) and biogeographic province (d). Error bars indicate standard errors. Different letters indicate differences between dung types or biogeographic provinces.

9 General discussion

Konik horses at the Zwindunes and polders, Knokke, Belgium (summer 2016).

9.1 Potential costs and benefits of endozoochorous seed dispersal

In its most strict definition endozoochory is the dispersal of plant seeds by herbivores through ingestion. This multi-step process involves the ingestion of plant seeds, the transfer through the gastrointestinal tract of a (potentially) mobile herbivore and the egestion of seeds with dung. One can distinguish two types of plant species in the context of endozoochory: those that show obvious morphological characteristics that attract seed- or fruit-eating animals (e.g., fleshy fruits, colourful seeds or fruits), and those that have no obvious structures to promote endozoochorous dispersal. For the first group, no one doubts the relevance of endozoochory, for the second it does not speak for itself.

The dispersal mechanism is expected to be beneficial for the plant species, because (1) it enables long distance dispersal due to the potentially long distances travelled by herbivores and the relatively long retention times in the gastrointestinal tract; (2) seeds are more likely to be directed towards suitable habitats due to habitat preferences of the herbivore and (3) towards fertile microhabitats, with dung being a highly nutritious, though largely organic, substrate, which (4) (temporarily) covers potential competitors in the existing vegetation. Most of the dispersal steps are covered in literature (e.g., Cosyns et al. (2005a), Cosyns and Hoffmann (2005), Nathan et al. (2008), Pakeman (2001), Traveset et al. (2007)). It shows that there are generally large costs for most plants to 'use' endozoochorous dispersal as dispersal mechanism, although these costs depend strongly on species specific characteristics (Table 9.1). Some species seem to have hardly any costs; some are simply wiped out during the endozoochory process. I further elaborate costs and benefits in §9.2.4 in this chapter, using the specific results from the experiments performed in this thesis.

Table 9.1 - Non-exhaustive overview of potential costs and benefits of endozoochorous seed dispersal.

Potential (species dependent) benefits	**Potential (species dependent) costs**
• Potential breaking of dormancy of hard-coated seeds (scarification of seed coat, high body temperature, increased seed coat permeability, etc.) • Potential long distance dispersal • Potential directed dispersal • Fertile deposition microhabitat • (Temporary) suppression of competitors in the local deposition vegetation • Nutrient rich environment after dung mineralisation • Secondary *shallow* burial of seeds in the soil by paracoprid or telecoprid dung beetles • Protection from predators	• Viability impacting damage of seeds at mastication • (Partial) digestion of seeds during gastrointestinal passage • Early germination in the intestines due to seed coat scarification and elevated temperatures • Unsuitable deposition microhabitat (unfavourable moisture and/or nutrient conditions, …) • Increased seed consumption by granivores caused by seed concentration in dung pats • Increased competition due to seed concentration in the dung pat • Potential germination suppression due to phytotoxins in dung • Increased competition after dung decomposition (mineralisation) due to local fertilization effects • Secondary *deep* burial of seeds in the soil by paracoprid or telecoprid dung beetles • Uncertainty of consumption and, hence, dispersal • Damage caused by micro-organisms

9.2 Post-dispersal fate of endozoochorously dispersed seeds

Endozoochorous seed dispersal can only be considered successful in the case that seeds remain viable **after** each of the successive steps of seed uptake, mastication, digestion, and deposition. But what is needed next? Are they able to germinate and to establish; are they able to grow into adult, reproductive plants at their deposition site? Dispersal success can be regarded as a combination of quantitative and qualitative aspects (Schupp et al., 2010). The quantity of seeds dispersed from the pre-dispersal environment is defined by the amount of seeds available at a certain moment and location, and the probability of being ingested by herbivores. Whether seeds are likely to be ingested is a complex interplay of plant (e.g., palatability, seed release height) and animal traits (e.g., selective habitat use and diet choice). Once the first condition for endozoochorous dispersal is met and seeds are ingested, qualitative aspects of the dispersal vector and deposition site determine whether seeds remain germinable during gastrointestinal passage and are able to establish and grow to mature plants in the post-dispersal environment (see Figure 1.3 in the general introduction and Albert et al. (2015b), Schupp (2010), Wang and Smith (2002)).

9.2.1 Dispersal probability

In recent decades, extensive proof for the existence and relevance of endozoochorous dispersal in semi-natural landscapes has been provided through germination trials using field-collected dung (e.g., Cosyns et al. (2005a), Couvreur et al. (2005a), Malo and Suárez (1995a), Pakeman et al. (2002)). As such, these studies have provided a valuable estimate of the dispersal probability and endozoochorous dispersal success of many grassland species. Table 9.2 provides an overview of the different factors that determine seed dispersal success for each of the plant species used in the experiments in this thesis. Several aspects of the quality of the dispersal vector and the deposition site are studied extensively in chapters 2 to 6. As this thesis mainly focuses on the fate of seeds following dispersal, factors determining the probability of being ingested by herbivores, such as the availability of seeds, herbivore grazing behaviour, and the palatability of

plants, are just briefly discussed in chapter 6 where dung was sampled to check the presence of *Helianthemum nummularium* seeds. For completeness, an estimate of the dispersal probability of the entire set of plant species used in the experiments throughout this thesis has been made based on literature sources. A palatability index was calculated for each plant species using the method described by Bossuyt et al. (2005) which includes the combination of grazing frequency data collected in an observational study by Lamoot (2004) with distributional data of each of the grazed species in the Westhoek nature reserve. Further, the occurrence of each species in field collected dung was adapted from an extensive endozoochory study by Cosyns (2004) carried out in the same coastal grassland area as Lamoot's (2004) observational study. By cross-linking the intake of seeds with the output in dung collected in the same study area, an estimate of the dispersal probability is given for the studied plant species. Despite the scarcity of grazing observations of some of the test species, a relation seems to exist between palatability and occurrence in dung with unpalatable species being less likely to be found germinating in dung (Table 9.2). Besides the palatability of plants, the number of seeds available in a plant population is a decisive factor in determining seed dispersal probability (Bruun and Poschlod, 2006). Furthermore, many grassland species, including the set of test species used in this thesis, produce rather large quantities of small seeds. As a seed size/seed number trade-off exists (Harper et al., 1970; Leishman, 2001), producing more but smaller seeds is thought to be a simple strategy to increase the dispersal ability of the maternal plant without changing the reproductive effort (Eriksson and Jakobsson, 1999). Also, a positive relation between seed abundance in the landscape and seed output in dung exists (Bruun and Poschlod, 2006), which might explain the lack of seeds of rare plant species such as *Helianthemum nummularium* in field collected dung despite grazing observations (see chapter 6). Although the probability for producing adult plants varies with species-specific factors such as seed size (Westoby et al., 2002), it should be noted that each adult plant originates from one successfully germinated and established seed. Despite the fact that quantitative aspects certainly have an impact on seed dispersal patterns, the relevance of qualitative aspects, such as the seed's ability to survive the gastrointestinal environment and to germinate and establish in its post-dispersal environment, should not be underestimated when assessing seed dispersal success.

9.2.2 Dispersal environment: quality of the dispersal vector

During their journey through herbivore gastrointestinal tracts, seeds are subjected to a wide range of digestive actions that may scarify the seed coat or completely destroy seeds. Digestive processes can be roughly classified into three major groups according to their mechanical, chemical or thermal nature. Mechanical scarification involves the grinding action of teeth during ingestion and rumination (Cox et al., 1993) and the abrasion of seeds by the gastrointestinal wall (Razanamandranto et al., 2004). Forage containing seeds is furthermore subjected to a broad spectrum of chemical processes. In the abomasum (ruminants) or the stomach (hindgut fermenters), seeds are soaked in hydrochloric acid and exposed to proteolytic, amylolytic and lipolytic enzymes, while proteolytic and cellulolytic enzyme secreting bacteria may become attached to the seed surface when moving through the rumen and large intestine of ruminants, or the cecum and colon of hindgut fermenters (Dijkstra et al., 2005; Gardener et al., 1993b; Householder et al., 1993). The chemical abrasion of seeds might result in an increased mortality, especially if the seed coat was already damaged during the mastication process which enables acids and bacteria to reach and kill the exposed embryo (Jaganathan et al., 2016). Alternatively, increased germination might result from the chemical scarification of seed coats, especially for seeds exhibiting physical dormancy (Yu et al., 2014). The high body temperature can also affect the survival of seeds in the gastrointestinal tract and damage the seed coat. In some extreme cases, the elevated temperature and the moisture in the gastrointestinal tract even provide optimal germination conditions and may provoke the protrusion of the embryo through the seed coat whilst residing in the intestines (Janzen, 1981; Janzen, 1982a). Nevertheless, early germination in the gastrointestinal environment most often results in seed loss due to digestive processes interacting with the exposed soft tissues of germinated seeds (Janzen et al., 1985).

Plant species largely differ in their ability to cope with the digestive processes in the herbivore gastrointestinal tract. Seed traits such as shape, size, and thickness and structure of the seed coat largely determine endozoochorous dispersal success. While small and rounded seeds are more likely to survive the gastrointestinal environment (Albert et al., 2015a;

Mouissie et al., 2005b; Pakeman et al., 2002), a hard and impermeable seed coat may protect the embryo during chewing and gastrointestinal passage (Jaganathan et al., 2016). Physical dormancy, a specific type of dormancy caused by a water-impermeable seed coat in mature seeds, is a widespread trait in flowering species and has often been linked with endozoochory (Baskin and Baskin, 2001; Venier et al., 2012; Willis et al., 2014). Although physical dormancy has been found in 15 plant families (Baskin et al., 2000), Fabaceae, Cistaceae, Malvaceae, and Geraniaceae are the most prevalent plant families with physical dormancy in temperate grasslands. In our experiments, the mechanical and chemical scarification of seeds often enhanced germination, especially in Cistaceae and Fabaceae species, while elevated temperatures rather had the opposite effect in most species (see chapter 2 and Table 9.2). We should however also note that there was a time factor associated with the applied thermal treatments. The negative effects on germination were stronger when the duration of treatments was longer. Hence, a crucial potential determinant of the fate of seeds is the time seeds spend in the gastrointestinal environment (Jaganathan et al., 2016). If seeds are expelled after a short time period in the gastrointestinal tract, it is highly probable that seeds are defecated with an intact seed coat. Especially if seeds have an impermeable seed coat, seeds may consequently remain dormant, but viable. In the best case scenario, seeds are defecated with mildly scarified seed coats after staying an optimal period in the herbivore body and are able to benefit from the nutritious dung environment by germinating shortly after deposition. But if seeds remain too long in the gastrointestinal environment they might experience irreversible damage (Jaganathan et al., 2016). Seed passage time varies both with herbivore and seed traits. In general, small, smooth and rounded seeds have a shorter retention time and are more prone to be egested in an undamaged state (Albert et al., 2015a) while the presence of a slimy seed coat may protect seeds during gastrointestinal passage and decrease seed retention time (Hintze et al., 2013; Kreitschitz et al., 2016). Furthermore, the body size and digestion strategy of the dispersal vector is also of importance as seeds remain longer in the gastrointestinal tracts of large herbivores compared to small herbivores with shorter intestines (Cosyns et al. (2005b), but see the long residence times in medium-sized herbivores such as wild boar in Picard et al. (2015)). Irrespective of herbivore species and body size, diet preference is also known to affect digestion time with shorter retention times for easily digestible forage (Blackshaw and Rode, 1991). In contrast to

the higher seed loss caused by longer retention times in large herbivore gastrointestinal tracts, seed abrasion through contact by the gastrointestinal wall is likely to be substantially lower in comparison with the short and narrow gastrointestinal tract of small herbivores (Neto et al., 1987). Also, as chewing time and tooth size are negatively correlated, the number of seeds fatally crushed during mastication could be lower in larger herbivores (Pellew and Southgate, 1984).

The combined risks of mechanical, chemical and thermal scarification of seeds during gastrointestinal passage might, therefore, result in considerable seed loss. Decreased and delayed germination during this first step of endozoochory has been demonstrated for a wide range of temperate grassland species using simulated digestion experiments (see chapter 2), and in controlled feeding experiments (see the germination of fed seeds of *Helianthemum nummularium* in chapter 6 and several literature sources, e.g., Cosyns et al. (2005b), D'hondt and Hoffmann (2011), Grande et al. (2013), Pakeman and Small (2009), Mancilla-Leytón et al. (2012)), although in some cases the opposite effect was found (e.g., Peco et al. (2006a), some species in D'hondt and Hoffmann (2011), and *Urtica urens* in chapter 2). Although the first condition for successful endozoochorous dispersal is the survival of seeds during their journey through herbivore gastrointestinal tracts, the final fate of seeds is more complicated as it includes the subsequent steps of germination, growth, and maturation in their deposition environment.

Table 9.2 - Factors determining seed dispersal success for each species used in the experiments in this thesis with an indication of life strategies ('LS', with A= annual and P= perennial). Increased values are marked with '+', decreased values with '-', indifferent results with 'i', and data gaps with 'na'. Results obtained in this thesis (chapters 2-6) are marked in bold. Palatability ('palat') was determined using the relative availability of plant species and grazing observations of the large herbivores in the Westhoek nature reserve using the method described by Bossuyt et al. (2005) and the observational data collected by Lamoot (2004) (with P= palatable, i= indifferent results, U= unpalatable, U*= no grazing records, and na= not native in this area). The abundance of each species in herbivore dung was adapted from Cosyns (2004) of plant species germinating from dung sampled in temperate grasslands ('Dgerm'). Species abundance in dung was classified as R= rare (<1 % of all seedlings), F= frequent (<5% of all seedlings), and A= abundant (>5 % of all seedlings) according to their occurrence in a set of 977 dung samples (Cosyns, 2004). The quality of the gastrointestinal environment was experimentally assessed by germination trials. The germinability following simulated mechanical ('mech'), chemical ('chem') and thermal ('therm') scarification of seeds was compared with untreated seeds (see chapter 2) and a comparison of the germination of fed seeds and intact seeds was made using literature sources ('Ggerm', sources: a= Cosyns et al. (2005b), b= D'hondt and Hoffmann (2011), c= Grande et al. (2013), d= Russi et al. (1992), e= Thomson et al. (1990), f= Cardoso et al. (2008)), g= Pakeman and Small (2009), and ch6= conclusions from the feeding experiment of chapter 6). The quality of the deposition site was determined by measuring germinability ('germ'), growth, flower number ('flower'), and adult biomass ('mass') in dung (see chapters 3 and 4), and by quantifying the effects of competition on the establishment ('establ'), flower number ('flower') and adult biomass ('mass') of plants (see chapters 5 and 6).

		Dispersal probability		**Quality of dispersal vector**				**Quality of deposition site**						
				Gastrointestinal environment				**Dung substrate**				**Competition**		
Plant family and species name	**LS**	***palat***	***Dgerm***	***mech***	***chem***	***therm***	***Ggerm***	***germ***	***growth***	***flower***	***mass***	***establ***	***flower***	***mass***
Caryophyllaceae														
Stellaria media (L.) Vill.	A	P	R	*na*	*na*	*na*	*na*	**-**	*na*	*na*	*na*	*na*	*na*	*na*
Cistaceae														
Cistus albidus L.	P	*na*	*na*	**+**	**i**	**-**	- [c]	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Helianthemum nummularium (L.) Mill.	P	U*	R	**+**	**i**	**i**	- [a,ch6] + [b]	**-**	**+**	*na*	*na*	**-**	*na*	**i**
Tuberaria guttata (L.) Fourr.	A	*na*	*na*	**i**	**i**	**i**	*na*	**-**	**i**	**i**	**i**	*na*	*na*	*na*
Cyperaceae														
Carex acuta L.	P	U*	*na*	**i**	**i**	**i**	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Carex flacca Schreb.	P	P	R	**i**	**i**	**i**	- [b]	*na*	*na*	*na*	*na*	*na*	*na*	*na*

Table 9.2 continued.

				Quality of dispersal vector				Quality of deposition site						
		Dispersal probability		Gastrointestinal environment				Dung substrate				Competition		
Plant family and species name	**LS**	***palat***	***Dgerm***	***mech***	***chem***	***therm***	***Ggerm***	***germ***	***growth***	***flower***	***mass***	***establ***	***flower***	***mass***
Fabaceae														
Medicago arabica (L.) Huds.	A	U*	R	**+**	**i**	**i**	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Medicago lupulina L.	P	U*	R	**i**	**i**	**-**	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium arvense L.	A	U*	R	**i**	**i**	**-**	- [a]	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium campestre Schreb.	A	U*	R	**i**	**i**	**-**	- [a,b] + [d, e]	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium pratense L.	P	U*	R	**i**	**i**	**-**	- [a] + [b]	**-**	**+**	**+**	**i**	**-**	**+**	**+**
Trifolium repens L.	P	i	F	**i**	**-**	**-**	- [a] + [f]	**-**	**+**	**i**	**+**	**-**	**i**	**+**
Gentianaceae														
Centaurium erythraea Baumg.	P	U*	R	*na*	*na*	*na*	- [a]	**-**	*na*	*na*	*na*	*na*	*na*	*na*
Juncaceae														
Juncus bufonius L.	A	P	A	*na*	*na*	*na*	- [b]	**-**	**i**	**-**	**-**	*na*	*na*	*na*
Juncus effusus L.	P	i	R	*na*	*na*	*na*	*na*	**-**	**i**	*na*	*na*	*na*	*na*	*na*
Poaceae														
Agrostis capillaris L.	P	U*	A	*na*	*na*	*na*	- [a,b]	**-**	**+**	*na*	*na*	*na*	*na*	*na*
Agrostis stolonifera L.	P	P	F	*na*	*na*	*na*	- [b]	**-**	**+**	*na*	*na*	**-**	**i**	**+**
Alopecurus myosuroides Huds.	A	U*		*na*	*na*	*na*	*na*	**-**	**i**	**i**	**+**	*na*	*na*	*na*
Poa annua L.	A	P	F	**i**	**i**	**i**	- [b]	**-**	**i**	**i**	**i**	*na*	*na*	*na*
Poa pratensis L.	P	U	R	**i**	**i**	**i**	- [a,b]	**-**	**i**	*na*	*na*	*na*	*na*	*na*
Urticaceae														
Urtica dioica L.	P	P	A	**i**	**i**	**i**	+ [g]	**-**	**i**	**i**	**i**	*na*	*na*	*na*
Urtica urens L.	A	U*	R	**+**	**+**	**+**	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*

9.2.3 Post-dispersal environment: quality of the deposition site

Dung as a substrate

Germination of plant seeds in the dung environment, which is typically moist and nutrient-rich in fresh depositions, often differs from a dung-free environment with more and faster germination in some cases (e.g., Archer and Pyke (1991), Carmona et al. (2013), Malo and Suárez (1995a), Quinn et al. (1994), Traveset et al. (2001)), or inhibited germination in others (e.g., Carmona et al. (2013), Izhaki and Ne'eman (1997), Paré et al. (1997)). Furthermore, the nutritive environment potentially affects the later stages in the plant life cycle, such as establishment and (seedling) growth (Bakker and Olff, 2003; Carmona et al., 2013; Cosyns et al., 2006; Traveset et al., 2001). In our experiments, dung had a clear negative effect on germination probability and timing compared with the dung-free control treatments (see chapter 3 and Table 9.2). This effect was even more pronounced in case cattle dung was applied. On the other hand, juvenile growth was hardly affected by the presence of dung while growth and flowering generally increased (see chapter 4 and Table 9.2).

Herbivore dung contains high concentrations of growth promoting macronutrients (N, K and to a lower extent P, Ca and Mg) and essential trace minerals (e.g., Fe, Mn and Cu) (Haynes and Williams, 1993; Lupwayi et al., 2000), and can serve as a natural fertilizer with larger plants as a result (Mancilla-Leytón et al., 2012). However, at the moment of defecation, only a small fraction of the nutrients present in the fresh dung pat is inorganic and most of the organic compounds must be mineralized before becoming available for plants (Jørgensen and Jensen, 1997; Sitters et al., 2014). The leaching of nutrients from dung to the underlying soil has been described in many ecosystems (e.g., Aarons et al. (2004), Haynes and Williams (1993)), although the rates at which nutrients are released are highly variable. Dung decomposition depends on factors such as humidity (Dickinson and Craig, 1990; Dickinson et al., 1981), chemical composition of dung (Ouédraogo et al., 2004), vegetation type of deposition sites (Shepherd et al., 2000), but also on the presence and activity of dung fauna such as dung beetles (Lovell and Jarvis, 1996; Yokoyama et al., 1991) and the occurrence of coprophilous fungi (Masunga et al., 2006; Richardson, 2001).

Despite the nutritive properties of dung, germination of the tested grassland species was reduced in cattle and horse dung. In addition to the high content of growth-promoting macro- and micro-nutrients, animal excrements contain small concentrations of growth-inhibiting substances such as phenolic compounds and fatty acids (Cosyns et al., 2005b; Malo and Suárez, 1995b; Marambe et al., 1993; Ramos-Font et al., 2015; Welch, 1985). These phytotoxic compounds may alter the activity of enzymes that regulate germination rate and, therefore, inhibit germination of certain plant species.

In addition to these phytotoxic compounds which are naturally present in dung, pollution with residues of anthelmintic drugs may also have a direct negative impact on the germination of grassland seeds (Eichberg et al., 2016). In an experimental assessment of the germinability of fed seeds of temperate grassland plants, Eichberg and colleagues (2016) found a clear negative effect of the presence of the commonly used anthelmintic formulation Cydectin and its active ingredient moxidectin on seed germination. It therefore seems that besides the indirect effects of anthelmintic drugs on plant regeneration through reduced functional diversity and abundance of dung beetles and their dung decomposing behaviour (Beynon, 2012; Beynon et al., 2012; Strong et al., 1996), veterinary drugs can also have a direct effect on plant population dynamics. Furthermore, viable seeds deposited in dung can be destroyed by a wide range of biotic and abiotic factors, including insects, fungi, rodents and desiccation (Estrada and Coates-Estrada, 2002; Vander Wall and Longland, 2004) and may even result in the complete disappearance of undigested soft seeds (Neto and Jones, 1986). Besides being a growth substrate for seedlings, dung can also promote the growth of fungi and bacteria which in some cases are detrimental to germination and seedling survival (Clark and Wilson, 2003; Traveset et al., 2007). Although high concentrations of nutrients leach from dung pats from the early phases of decomposition onwards, on the condition that sufficient moisture is present (Dickinson and Craig, 1990), the presence of nutrients is probably not decisive in the early developmental phases of seedlings as many seeds contain a reserve of mineral and organic nutrients to nourish the embryo in its initial stages of establishment (Fenner and Thompson, 2005). Once this storage is depleted seedlings have to use the available nutrients in the soil, and the

accumulated nutrients leached from the decomposing dung pat become useful to endorse growth and flowering.

Dung originating from different herbivore species may differ in its chemical and structural composition, but is also linked to herbivore diet (e.g., the differing diets of stabled versus free-ranging animals) and the nutritive physiology of mammals (e.g., ruminants vs. non-ruminants) (Holter, 2016). On average, the dung from non-ruminant species is more fibrous and has a lower nitrogen content compared with ruminant dung (Holter, 2016). This nutritive effect was also found in the higher growth rates of some of our tested species grown in cattle dung. On the other hand, fewer seeds were able to germinate in cattle dung compared with horse dung, and it, therefore, seems that the initial cost of reduced germination is compensated by a lush growth in the later growth stages. Furthermore, the structural composition may have a large impact on the post-dispersal fate of seeds. Dung types consisting of small pellets (such as sheep, deer and rabbit excrements) may desiccate rapidly and prevent the imbibition of seeds prior to germination (Eichberg et al., 2007; Welch, 1985). Moreover, the surface layer of watery dung types (such as cattle dung) may transform into a hard and dry crust soon after deposition. Therefore, many dung types may become a very dry environment which is suboptimal for germination and seedling growth. Although the high moisture content of fresh dung can promote early germination of fast-germinating species (Traveset et al., 2001), the imbibition of slower germinating species is impeded by the desiccation of the surface layers (Brown and Archer, 1989). The disintegration of dung pellets through trampling of grazing animals or rainfall, and the decomposition by coprophilous insects and fungi may be of utmost importance for the establishment of certain plant species that cannot emerge from intact dung (Mancilla-Leytón et al., 2012).

Besides the impact of the chemical and structural composition of herbivore dung, the growth response of seeds sown in dung may differ among plant species. Carmona and colleagues (2013) measured longer roots in species known to increase their distribution under grazing (*i.e.* grazing increaser species) when dung leachates were provided, while the opposite effect was found in grazing decreaser species. The normally rare species *Helianthemum nummularium* which we studied in chapter 6 could be classified as a grazing increaser species as its distribution increased remarkably after the introduction of large herbivores in calcareous coastal dune grasslands in

Belgium (see Provoost et al. (2015)). Although a low N-indicator value (Ellenberg, 1974), has been assigned to this species and this species can be hardly considered as nitrophilous, the cover of *H. nummularium* is known to increase at the edge of cattle dung patches (Dai, 2000). This observation is in line with the increased height and growth rate found in our growth experiment in chapter 4 and is probably related with endozoochorous dispersal.

The deposition of dung may have a profound effect on competitive interactions between plant species. An indirect effect is the deposition of dung itself as fresh dung often suppresses the existing vegetation and consequently creates a beneficial microhabitat for germination by eliminating competition with the already developed vegetation (Traveset, 1998). This is especially the case for dung deposited in large and dense volumes, such as cattle and horse dung, which often smothers and kills the underlying vegetation due to the lack of light during the decomposition period (Traveset, 1998; Williams and Haynes, 1995). It results in the creation of temporary gaps with relatively low levels of competition between seedlings and the established vegetation (Brown and Archer, 1989). Furthermore, large depositions of dung are usually avoided by grazing herbivores (Castle and MacDaid, 1972) and hence endozoochorously dispersed seeds could benefit from reduced competition with the existing vegetation, lowered grazing risks and the nutrients that are present in the dung pat. Therefore, dung pats create islands or safe sites in the existing vegetation for the germination and establishment of endozoochorously dispersed seeds.

Establishment in a competitive environment

Previous studies have found high concentrations of germinable seeds in dung depositions of large herbivores (e.g., Cosyns et al. (2005a), Cosyns and Hoffmann (2005), Malo and Suárez (1995b)). Although the content of germinable seeds in dung varies with seed availability in the environment, herbivore size and type, and season, the high variability in seed concentrations and species combinations suggest that seed dispersers not only affect plant fitness by the act of dispersal itself, but also by the different combinations and densities of the seeds deposited with their dung

(Loiselle, 1990). We found a negative correlation between seed density and establishment success although the magnitude differed between species combinations (see chapters 5 and 6). Despite the fact that we were limited in the number of species combinations due to practical constraints, we can conclude that, at least for the tested species, the cost of endozoochorous dispersal increases with increasing seed densities. Therefore, a selective pressure may have led to the evolution of strategies to overcome the negative effects of competition in these seed clumps. When seeds end up in a competitive environment, plant species may adapt their own germination timing or inhibit the germination of conspecific or heterospecific neighbouring seeds by producing allelopathic chemicals (Dennis et al., 2007; Greer et al., 2014; Murray, 1998). Through shorter germination times, or '*adaptive acceleration*' (Turkington et al., 2005), seeds avoid resource competition with later emergents at high densities (Dyer et al., 2000). Early-germinated plants have more time to grow in an environment with moderate competition and generally grow faster and larger compared to seeds germinating later (e.g., Bergelson and Perry (1989), Black and Wilkinson (1963)). Another strategy is to avoid competition by delaying germination until the competition is less fierce ('*adaptive delay*'). This strategy is mostly found in species with dormant seeds, and might be relevant in *Helianthemum nummularium* as the germination timing of this species can be very long and unpredictable, even after gastrointestinal passage (see the results of the feeding experiments in chapter 6). In our experiments, the presence of dung was the most powerful determinant of establishment success and had a negative effect on the early life stages of plants, especially in high seed densities. Once established, plants in low seed densities generally grew faster, produced more flowers, and had higher biomass when grown in dung (chapter 5). As the outcome of the competitive interactions differs among species combinations, dung pat islands may trigger a shift in species co-existing patterns through the combination of reduced competition with the established vegetation, altered competitive processes within the dung pat and the influx of seeds.

9.2.4 Costs and benefits of endozoochory, what did we learn from our experiments?

The costs associated with the dispersal of organisms can be broadly classified into 4 groups including direct energetic, risk and time costs and indirect opportunity costs (Bonte et al., 2012). Plants, as sessile organisms, have rather limited dispersal options. Reproductive structures such as seeds, spores, or fruits are the prevailing dispersal propagules, although vegetative structures such as bulbils, rhizomes or other plant parts can serve as dispersal units as well (Poschlod et al., 2005). Unlike animals which generally invest energy in the development of dispersal structures and the movement itself, *energetic costs* in plants are mostly limited to the production of dispersal units adapted to a certain dispersal method, such as palatable fruits, seeds containing wings, hooks or bristles or seed coat structures resisting gastric fluids. Since these morphologic adaptations are made during the developmental phase, these energetic costs are paid by the plant regardless of whether its seeds get dispersed. In theory, plants investing more in dispersal structures pay a higher price in case they do not get dispersed, but this is rather hard to quantify and lies beyond the scope of this thesis. Therefore, energetic costs are not taken into account in the cost-benefit calculation, although we should keep in mind that within plant species the energetic investment in reproductive organs may differ.

Risk costs are probably the most studied costs of endozoochory and include the loss of seeds due to the digestive processes during the transfer phase and reduced germinability at the deposition site due to the presence of dung or density related seed competition. While the presence of dung imposed a risk cost due to the lowered germination success of all tested species in our experiments, the effects of (simulated) gastrointestinal passage were less straightforward (Table 9.3). Especially the thermal aspects of the passage through the gastrointestinal tract led to reduced germinability whereas certain Cistaceae, Fabaceae and Urticaceae species benefitted from the mechanical scarification of the seed coat caused by the grinding action of the denture. Instead of paying a risk cost with the irreversible loss of seeds, one particular species, *Urtica urens*, even experienced a net benefit in this step of the dispersal cycle as a higher proportion of seeds germinated due to gastrointestinal treatment. Furthermore, the germinability of most other species was left unchanged

implying that no risk costs were charged during gastrointestinal transfer for these species.

Dispersal may also represent a *time cost* which refers to the direct costs due to the time invested in the movement itself that cannot be invested in other activities, such as germination (Bonte et al., 2012). However, the time lost during dispersal is rather arbitrary in the case of endo- or epizoochorous dispersal as in most plant species a germination delay of a few days presumably is not decisive for plant fitness. On the other hand, the time spent in the gastrointestinal tract is a crucial determinant of seed fate as it is closely linked with seed survival probability (Jaganathan et al., 2016) and herbivore species (Cosyns et al., 2005b) and diet (Blackshaw and Rode, 1991). Nonetheless, Cosyns et al. (2005b) found different gastrointestinal passage rates and seed survival among different individuals of the same herbivore species although the animals were fed with the same forage.

The fourth group of costs is related to the post-dispersal environment, which may reduce the fitness of the dispersed organism. These *opportunity costs* include the dispersal towards less suitable habitats or the loss of the advantages of adaptations to a certain habitat. Certain aspects of these opportunity costs are time-related and include the timing of life cycle events of dispersed plants such as the timing of germination, the growth rate of established plants and the timing of flowering. When seeds are deposited in large quantities as is often the case in endozoochory, it may be beneficial for seeds to germinate early to beat the rush or to wait out the crowd as dormant seeds (Dennis et al., 2007). Through the incorporation of dormant seeds due to endozoochorous dispersal, soil seed banks may get supplied by a fresh seed load and become more diverse in species composition (Dai, 2000; Nathan and Muller-Landau, 2000). Early emergence may be advantageous in an environment with strong competitive forces and in certain cases even a difference in germination timing of a few days may decrease the growth rate and survival probability of later emerged seedlings (Loiselle, 1990; Traveset, 1998). On the other hand, selection may not always be in favour of early germination as early germinated seeds are often more susceptible to pathogens, seed predators and adverse environmental conditions (Janzen, 1984; Traveset, 1998). Furthermore, the activity of dung-inhabiting fauna whose dung removing and mixing actions may harm vulnerable seedlings decreases with ageing dung (Janzen, 1984). As dung ages, a higher concentration of the nutritive content of dung is mineralized

and becomes available for plant uptake, which is beneficial for the growth of seedlings that germinated later (Jørgensen and Jensen, 1997; Sitters et al., 2014). Therefore, the ideal germination timing after endozoochory is rather a trade-off between the reduced competition early after deposition and the higher availability of nutrients in a later stage. Furthermore, **t**he post-dispersal environment may have a profound effect on plant fitness due to the high nutrient content (Jørgensen and Jensen, 1997). In our experiments, we mainly found beneficial effects of the nutritious dung environment which were reflected in higher and heavier plants with a higher reproductive output (Table 9.3). In the trade-off between costs and benefits of our studied species, opportunity costs largely corresponded with the patterns of risk costs in the first phase of the plant life cycle with faster germination of certain plant species following simulated gastrointestinal passage and slower germination in dung (Table 9.3). Furthermore, these initially high costs of delayed germination were in most species compensated in later phases with faster growth rates and accelerated flowering of plants growing in dung. Also, we should note that our experiments spanned only one growing season and possible beneficial effects of delaying germination to the next year.

Although the costs of losing seeds during gastrointestinal passage can be very high for many species (Cosyns et al., 2005b; Grande et al., 2013; Mancilla-Leytón et al., 2012; Pakeman and Small, 2009), the post-dispersal costs are moderate for many of our tested species. Also, the presence of dung did not affect the further development of some species which suggests that endozoochorous dispersal is rather cost efficient for these indifferent species, at least in the later developmental stages. Furthermore, the mortality costs of undispersed seeds might also be high. Most grassland species produce vast amounts of seeds which would result in even higher competition levels in the vicinity of the mother plant. Herbivores may serve as a filter for seeds through the differential survival of seeds in their gastrointestinal tracts whereas undispersed or wind-dispersed seeds would go through an environmental filter instead (Cavallero et al., 2012).

We should also keep in mind that our germination, growth and competition experiments were mainly performed in laboratory (chapter 2) or greenhouse conditions (chapters 3-6). The more controlled environment with more favourable combinations of light quality, day-night cycle length, and more constant temperatures and humidity might result in more or

faster germination and growth in comparison with field conditions (see chapter 3 and Ramos-Font et al. (2015)). Therefore, the effects of dung on germination, growth and competition might be even more pronounced in real-life field conditions. Furthermore, we used intact seeds in the germination, growth and competition experiments in chapters 3 till 6 due to practical considerations and in an attempt to reduce complexity. Thus, the measured effects of the presence of dung or seed density should be regarded as the outcome of just one single step in the endozoochorous dispersal process, and the full costs of endozoochory might be higher.

9.2.5 Implications for vegetation ecology and nature conservation

Dispersal patterns of seeds and pollen have a large impact on the genetic diversity, spatial distribution and composition of plant communities (Wang and Smith, 2002). Although gene flow in and between plant populations can also be mediated by the dispersal of pollen, dispersal rates and distances, and hence the effectiveness of gene transfer, may differ between seeds and pollen (Ennos, 1994; García et al., 2007). Although pollen is generally able to cover larger distances compared to unassisted or scatter-hoarded seeds (Ennos, 1994), more long-distance dispersal events occur when seeds are dispersed by vertebrate herbivores (García et al., 2007). These long-distance dispersal events may play a major role in various ecological and evolutionary processes and have had a critical impact on plant communities through processes as the colonization of islands, Holocene migrations, metapopulation biology and in providing a response to global change (Cain et al., 2000; Nathan, 2006; Pakeman, 2001). Especially in fragmented landscapes, such as today's cultural landscapes in temperate Europe, dispersal limitation imposes one of the main obstacles for the restoration of species-rich grasslands (Bakker and Berendse, 1999; Ozinga et al., 2009). From the start of the industrialisation period onwards, traditional and small-scale agropastoral practices have been disrupted (Eriksson et al., 2002). The combination of fragmentation and land-use intensification has led to a dramatic loss and change of seed dispersal processes and vectors in anthropogenic landscapes. The reinstatement of traditional land use practices could, therefore, restore some of the dispersal processes and maintain a permanent seed flow between similar habitats (Poschlod and

Bonn, 1998). In this context, free-ranging domestic herbivores, such as cattle, horses and sheep, are commonly introduced as a nature management measure in order to counteract further deterioration of the highly fragmented, isolated and small nature areas in Europe. Through their differential grazing behaviour and the selection of particular species and habitats, these herbivores help to create spatial heterogeneity and to prevent shrub encroachment (Adler et al., 2001; Cosyns and Hoffmann, 2005; Lamoot et al., 2005b; Olff and Ritchie, 1998). Besides this direct impact on vegetation structure and composition, the indirect impact of grazing through the zoochorous dispersal of seeds is as important in nature conservation. Although domestic ungulates are able to increase species richness on a local scale through the internal (e.g., Cosyns et al. (2005a), Eichberg et al. (2007), Traba et al. (2003)) or external (e.g., Chuong et al. (2016), Couvreur et al. (2004a), Manzano and Malo (2006)) transport of high quantities of seeds, their home ranges are often relatively small due to the fragmentation of agropastoral landscapes. The impact of seed dispersal by wild herbivores might differ from captive animals due to their larger home ranges and different behaviour and habitat use (Malo et al., 2000; Pellerin et al., 2016). Several deer species, wild boar, rabbits and hares have been used as focal organisms in endozoochory (see e.g., Eycott et al. (2007), Pakeman et al. (1999), Pellerin et al. (2016), Picard et al. (2016)) and epizoochory studies (see e.g., Heinken and Raudnitschka (2002), Picard and Baltzinger (2012)). The dispersal of a wide range of plant species by wild herbivores has been recorded, although seed dispersal efficiency largely differed among disperser species which could be attributed to differences in fur structure (Heinken and Raudnitschka, 2002; Picard and Baltzinger, 2012), feeding habitat and diet choice (Eycott et al., 2007; Pellerin et al., 2016) and functional traits of the dispersed plant species (Picard et al., 2016). Furthermore, as wild herbivores are in theory not restricted to one particular area (in contrast with the introduced domestic herbivores which are confined to one particular fragment), movements between similar patches could increase habitat connectivity through zoochory. Due to the different habitat use and diet choice, seed dispersal by domestic ungulates and wild, native herbivores is often complementary. In this regard, it is vital to conserve and restore seed dispersal processes in an attempt to improve connectivity between isolated plant populations (Pywell et al., 2002), restore degraded habitats (Freund et al., 2014) and to cope with land use and climate change.

Table 9.3 - Summary of the types of costs and benefits of endozoochorous seed dispersal measured for each species studied in this thesis. Costs are marked with 'C', benefits with 'B', indifferent results with 'i', and data gaps with 'na'. Risk costs evaluate the germinability of seeds following simulated mechanical ('mech'), chemical ('chem') and thermal ('therm') scarification (see chapter 2) or seeds sown in dung (column 'dung', see chapter 3), and the establishment of seedlings with inter- and intraspecific competition ('Establ. comp., see chapter 5 and 6). Risk costs include increased mortality of seeds and competing seedlings (C) and the breaking of dormancy or the winning of the competition process (B). Opportunity costs include the time needed to germinate following simulated mechanical ('mech'), chemical ('chem') and thermal ('therm') scarification (see chapter 2) or when seeds are sown in dung (column 'dung', see chapter 3), the growth rate of seedlings in dung (column 'Gr. rate'), the time first flowers appear (column 'Flower timing'), the height of adult plants ('Height (ad.)'), the maximum flower number ('Flower No.') and biomass of flowering plants ('Biomass (fl. ad.)') sown in dung (see chapter 4 and 5). Increased time, height, flowering and biomass were considered as a benefit (B), decreased values as a cost (C).

Types of costs and benefits	**Risk**					**Opportunity**								
	Germination				**Establ.**	**Germination**				**Gr.**	**Flower**	**Height**	**Flower**	**Biomass**
Plant family and species name	***mech***	***chem***	***therm***	***dung***	**comp.**	***mech***	***chem***	***therm***	***dung***	**rate**	**timing**	**(ad.)**	**No.**	**(fl. ad.)**
Caryophyllaceae														
Stellaria media	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	*na*
Cistaceae														
Cistus albidus	B	i	C	*na*	*na*	B	i	C	*na*	*na*	*na*	*na*	*na*	*na*
Helianthemum nummularium	B	i	i	C	C	B	C	C	C	B	*na*	B	*na*	*na*
Tuberaria guttata	i	i	i	C	*na*	i	i	C	C	i	i	i	i	i
Cyperaceae														
Carex acuta	i	i	i	*na*	*na*	i	C	i	*na*	*na*	*na*	*na*	*na*	*na*
Carex flacca	i	i	i	*na*	*na*	B	B	B	*na*	*na*	*na*	*na*	*na*	*na*
Fabaceae														
Medicago arabica	B	i	i	*na*	*na*	B	B	i	*na*	*na*	*na*	*na*	*na*	*na*
Medicago lupulina	i	i	C	*na*	*na*	B	B	C	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium arvense	i	i	C	*na*	*na*	i	i	C	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium campestre	i	i	C	*na*	*na*	C	i	C	*na*	*na*	*na*	*na*	*na*	*na*
Trifolium pratense	i	i	C	C	C	i	i	C	C	i	i	B	B	i
Trifolium repens	i	C	C	C	B	i	C	C	C	i	B	B	i	B

Table 9.3 continued.

Types of costs and benefits	Risk					Opportunity								
	Germination				Establ.	Germination				Gr.	Flower	Height	Flower	Biomass
Plant family and species name	*mech*	*chem*	*therm*	*dung*	comp.	*mech*	*chem*	*therm*	*dung*	rate	timing	(ad.)	No.	(fl. ad.)
Gentianaceae														
Centaurium erythraea	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	*na*
Juncaceae														
Juncus bufonius	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	B	C	i	C	C
Juncus effusus	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	i	*na*	i	*na*	*na*
Poaceae														
Agrostis capillaris	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	i	*na*	B	*na*	*na*
Agrostis stolonifera	*na*	*na*	*na*	C	C	*na*	*na*	*na*	C	i	*na*	B	*na*	*na*
Alopecurus myosuroides	*na*	*na*	*na*	C	*na*	*na*	*na*	*na*	C	i	C	i	i	B
Poa annua	i	i	i	C	*na*	i	B	C	C	i	i	i	i	i
Poa pratensis	i	i	i	C	*na*	B	B	C	C	i	*na*	i	*na*	*na*
Urticaceae														
Urtica dioica	i	i	i	C	*na*	i	i	C	C	i	i	i	i	i
Urtica urens	B	B	B	*na*	*na*	B	B	B	*na*	*na*	*na*	*na*	*na*	*na*

9.3 Secondary seed dispersal and dung removal by dung beetles

9.3.1 Dung beetle diversity

Spatial distribution

The functional composition and species diversity of dung beetle assemblages is driven by various factors. On a large spatial scale, climate and biogeographic factors mainly determine dung beetle assemblage composition. In Europe, abundance, species diversity, functional composition and habitat specialisation of dung beetles differ along a latitudinal range. In general, fewer species and lower dung beetle abundance are found with increasing latitudes. The functional composition of dung beetle assemblages varies between biogeographic regions with a shift from dweller dominated assemblages in the northern zone towards more diverse assemblages composed of dwellers, tunnelers and rollers in the south (Cambefort, 1991; Verdú and Lobo, 2008). Furthermore, habitat and resource specialisation differs along a north-south gradient with more generalists being found in the north. The current distribution of dung beetle richness largely traces back to the post-glacial dispersal patterns (Hortal et al., 2011). These general patterns of dung beetle assemblage composition were confirmed in the multi-site experiment in the Western Palaearctic region (chapter 8). In addition to the north-south gradient, species assemblages differed also along a west-east gradient which was associated with temperature conditions. As dung beetles most probably originated in the warm tropical climates during the Mesozoic (Davis et al., 2002) and the current distribution of Western Palaearctic dung beetle assemblages is closely linked with the location of the current and last-glacial location of the 0 °C isotherm (Hortal et al., 2011), thermal conditions may be strictly regulating species compositions. Considering the ongoing climate change, this temperature-diversity relationship might become increasingly important in ecosystem ecology. In the current climate models for the European continent, the continental interior of Eastern Europe will warm more rapidly during winter, whereas in summer the pattern of warming follows a strong south-to-north gradient with South Europe warming at a much faster rate than North Europe (IPCC, 2014). In the multi-site experiment particularly high species richness was found in Eastern Europe (e.g., in the Hungarian

study sites in the Pannonian and Middle European forest ecoregions) while dung beetle assemblages in South Europe were less diverse (e.g., the Spanish and French study sites in the Iberian highlands and Mediterranean sclerophyl ecoregions respectively). Research in temperate and Mediterranean Europe highlighted that the current dung beetle distribution is largely defined by minimum temperatures and the failure of most species to go beyond their specific temperature range limits (Lobo et al., 2002; Menéndez and Gutiérrez, 1996; Menéndez and Gutiérrez, 2004). Furthermore, Menéndez and Gutiérrez (2004) found a temperature-driven shift in habitat associations of dung beetle species in northern Spain with a preference for closed habitat types in the warmest season and more open habitats in winter. Global change might therefore not only result in more diverse dung beetle assemblages in the north and lowered diversity in the south as predicted by Dortel et al. (2013) but could also trigger a shift in habitat selection on a regional scale. As the response of dung beetle species to a changing climate differs largely between species (Menéndez and Gutiérrez, 1996; Menéndez and Gutiérrez, 2004), climate change could generate new species assemblages and change species interactions.

Threats to dung beetle diversity

Recently, concerns have risen regarding the decline in species and functional richness of dung beetles in Europe (Carpaneto et al., 2007; Dortel et al., 2013). Especially the abundance and diversity of roller and large bodied species has gradually decreased in recent decades. For example, in the Mediterranean region of southern France and Spain, a marked decline in roller diversity and abundance has been recorded since the 1950s (Lobo, 2001; Lobo et al., 2001) and in the Padana Plain in Northern Italy at least 4 roller species have gone regionally extinct in the same era (Barbero et al., 1999). In Finland, Rosenlew and Roslin (2008) found a significant loss of large tunnelling Geotrupidae beetles (with one out of three species being regionally extinct at present) and small tunnelling *Onthophagus* species (with the loss of two out of three species). This decline in dung beetle diversity has probably been caused by a combination of triggers related to changes in human land use (Barragán et al., 2011) such as the cessation of small-scale cattle and sheep herding in many European regions (Barbero et al., 1999), the increasing urban development in the Mediterranean coastal

areas (Carpaneto et al., 2007; Lobo, 2001), and the ubiquitous use of anthelmintics and other veterinary drugs in livestock farming (Beynon, 2012; Jochmann and Blanckenhorn, 2016; Strong et al., 1996). Furthermore, the intensification of pasture management and the decline of extensively grazed pasture area have likely contributed to the decline and regional extinction of dung beetle species (Hutton and Giller, 2003; Jay-Robert et al., 2008; Söderström et al., 2001). Extensively grazed pastures represent an important habitat for dung beetles in today's fragmented landscapes in Western Europe (see box 9.1, Hutton and Giller (2003)) and organic farming systems with limited application of (chemical) fertilizers and veterinary drugs may, therefore, play an important role in biodiversity conservation (Hole et al., 2005; Hutton and Giller, 2003). Furthermore, the current dung beetle diversity dates back to the historic land use of pasture areas. Despite the fact that dung beetles are good dispersers and inhabit ephemeral resources, local grazing continuity is of utmost importance for dung beetle diversity (Buse et al., 2015). Especially the abundance of habitat specialists and vulnerable species increases with pasture size and grazing continuity (Buse et al., 2015). On the other hand, the effects of vegetation structure on dung beetle assemblages have been the subject of debate. Whereas Buse et al. (2015) and Söderström et al. (2001) conclude that vegetation structure is only of minor importance for dung beetle species richness and assemblage composition, Hutton and Giller (2003) positively related dung beetle diversity with structurally more diverse pastures. Furthermore, in abandoned and encroached pastures in the north-western Italian Alps, dung beetle diversity rapidly increased after the reinstatement of pastoral practices due to the removal of shrubs (Tocco et al., 2013). Therefore, the impact of grazing is determined to a large extent by local ecological conditions and the biogeographical context that has shaped the composition of communities over time (Barragán et al., 2014).

Box 9.1 How does life-stock management affect dung removal rates and dung beetle assemblage structure? A case-study comparing intensively and extensively grazed grassland in Belgium

Introduction

Dung beetles provide some key ecosystem functions which are closely related to the manipulation of dung in their feeding and nesting process. While burying dung underground, dung beetles have direct effects on nutrient cycling and bioturbation, and indirectly on pest control, secondary seed dispersal and plant growth (Hanski and Cambefort, 1991b; Nichols et al., 2008). Due to this extensive list of functions, dung beetles can be considered as vital ecosystem engineers in natural ecosystems, but also as an asset in agriculture, both for crop and livestock farming. A great economic value has been attributed to dung beetles. Although it is difficult to determine an exact value, the annual economic value of the services of dung beetles in forage production, dung removal, and parasite suppression has been estimated to at least 380 million dollars in the United States. Losey and Vaughan (2006) acknowledged that this value most probably is an underestimate while Beynon et al. (2015) have put a value of 367 million pounds on dung beetle ecosystem services in the United Kingdom alone. No such figures are available for Belgium, but as the agricultural industry comprises 2.5 million heads of cattle in Belgium (AD Statistiek - Statistics Belgium, 2015) and knowing that each animal can produce over 9000 kg of solid waste per year (Fincher, 1981), we can safely assume that dung beetles represent a significant economic value for the cattle industry in Belgium.

Despite the great ecological and economic value of dung beetles, the intensification of modern cattle farming negatively affects dung beetle diversity and abundance. In an assessment of the impact of farm management, Hutton and Giller (2003) found lower abundance, biomass, diversity and species richness in intensive and rough grazing farms compared to organic farms. Although land use changes and the abandonment of pastoral practices are known bottlenecks for dung beetle diversity (Barragán et al., 2011; Carpaneto et al., 2007; Lobo, 2001; Roslin and Koivunen, 2001), the intensification of agriculture has had a similar effect (Barbero et al., 1999; Negro et al., 2011). The use of chemical fertilisers, veterinary drugs, the removal of herbaceous field boundaries and the lack of structural diversity in the pastures is most probably causing the decline in dung beetle richness (Hutton and Giller, 2003).

In 2016, a multisite experiment was conducted in cooperation with a large consortium of international researchers in 37 countries around the world. This multisite experiment was conceived and coordinated by Jorge Ari Noriega (Natural Museum of Natural Science (CSIC), Madrid, Spain) and has been replicated at numerous pairs of intensively and extensively grazed grasslands. The main goals of

the project are to assess how cattle management intensity in grasslands affects dung removal rates in different biogeographic and climate regions around the world, how this is related to morphological traits diversity. In this box, the preliminary results of the Belgian part of the experiments are reported.

Materials and methods

Study sites

The experiments were run simultaneously in August 2016 at two grassland sites which are either intensively or extensively grazed by cattle. The intensively grazed site ('Hazegras', 51°19'27.7'' N; 3°20'6.2'' E) is grazed by 8 animal units/ha of Belgian blue and has been in agricultural use for centuries, while the extensively grazed site ('The Zwin', 51°21'21" N, 3°20'41" E) is part of the Flemish nature reserve 'Zwindunes and polders' since 2002 and is grazed by 0.5 animal units/ha of Highland cattle year-round. The study sites are located 3.5 kilometres from one another within the same altitudinal range (0-2 m a.s.l.) at the most north-eastern part of the Belgian coast (Figure 9.1).

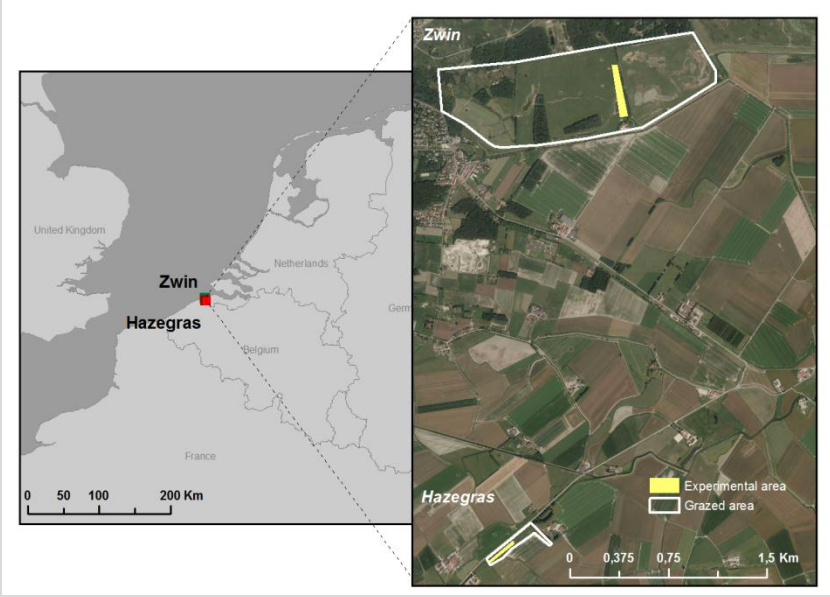

Figure 9.1 - Location of study areas within Belgium (left) and the extent of the grazed areas and the positioning of the experimental locations (right) in the extensively grazed area (Zwin) and intensively grazed area (Hazegras). The base layers used in the detailed map are aerial photographs of summer 2012 provided by the Flanders Geographical Information Agency. © AGIV (http://www.agiv.be/)

The Zwin site is a relatively species rich dune-polder transition grassland on a sand-clay soil with *Holcus lanatus*, *Alopecurus pratensis*, *Cynosurus cristatus*, *Agrostis capillaris* and *Lolium perenne* as the most abundant grass species, while the nearby

Hazegras site is a polder grassland on a clay soil dominated by *Lolium perenne*. The soil types of both areas can be classified as calcaric fluvisol (IUSS Working Group, 2006). Furthermore, the study sites are classified in the hydrogeological zone of 'Polders and silted areas' (Databank Ondergrond Vlaanderen (DOV), 2013) which indicates that the environmental parameters of groundwater level and quality are broadly comparable. Both study areas are located in the Atlantic climate zone (Cfb in the Köppen classification (Peel et al., 2007)) with an average annual precipitation of 722 mm, an average annual temperature of 10.1 °C and with averages of 79.3 mm and 18.0 °C in the month of the experiment.

Dung removal experiment

The experiment was part of a worldwide multisite experiment following a standardised field protocol in extensively and intensively grazed grassland (Noriega, 2016). At each study site, two transects with ten experimental units and five control units were installed. Experimental units were spaced 25 m from each other and the control units, while control units were at a distance of 50 m (Figure 9.2).

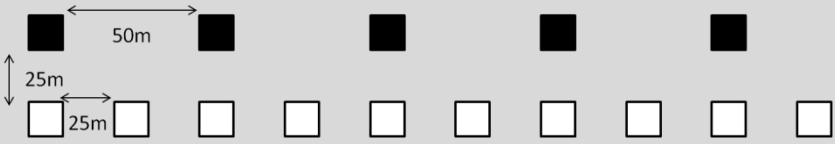

Figure 9.2 - Experimental design with 10 experimental units (white boxes) and 5 control units (black boxes).

Each experimental and control unit had a surface area of 0.5 m x 0.5 m which was sod-cut before starting the experiment. In order to use dung beetle free and non-toxic dung in the experiment, fresh dung was collected from stabled cattle which were not treated with any veterinary drugs for at least the last six months. The dung was homogenised and the same dung was used at both study sites in order to prevent interactions between dung removal rates and dung quality. To measure the wet: dry ratio of the dung, a sample of 300 g of fresh dung was dried for 72 h at 80 °C. At the start of the experiment, exactly 300 g of fresh dung was put in both the experimental and control units. Experimental plots were accessible to any kind of dung fauna, while the control plots were covered by plastic nets with a mesh size of 1 mm² (square mesh with side lengths of 1 mm) and the borders were dug into the soil for 5 cm in order to prevent the colonization of the dung pat by any creature flying or crawling towards it. After 48 h, the experiment was stopped and the remaining dung was collected from the units. Soil particles, as well as dung beetles, were removed from the dung using tweezers and a brush. The mass of the remaining dung was measured instantaneously. Afterwards, dung samples were dried during 72 h at 80 °C and their dry mass was measured.

Dung beetle sampling and measuring

In order to link dung removal rates with dung beetle diversity and abundance at both study sites, dung beetles were sampled at the moment the dung removal experiment was stopped. At each study site, ten dung baited traps were installed at the location of the experimental units. Each pitfall consisted of one plastic 1l container which was dug into the soil with the upper rim levelled with the soil surface. A saturated salt-water solution (ca. 365 g/l NaCl with some drops of unscented detergent) was used as a fixation fluid. Sampling pots were covered by chicken wire with a mesh size of 2 x 2 cm² and on top 300 g of fresh dung was put as an attractant for dung fauna. Traps were left in the field for 48 h. Scarabaeoidea were identified to species level using Janssens (1960) and Jessop (1986). Each species was assigned to a functional group according to its dung processing behaviour: endocoprids (dwellers), paracoprids (tunnelers) and telocoprids (rollers) (Doube, 1990). Ten individuals from each species were selected randomly to measure nine morphological body traits using a digital calliper with an accuracy of 0.01 mm. In case the species was found in both study sites, five individuals were selected from each site. If less than five individuals were found at a site all individuals from that site were measured and more individuals of the other site were measured to sum ten individuals of that species. Morphological traits measured were: head length (HL), head width (HW), pronotum length (PL), pronotum width (PW), pronotum height (PH), elytron length (EL), protibia length (pTL), protibia width (pTW) and metatibia length (mTL) (Figure 9.3). Afterwards, the measured individuals were oven dried at 80 °C during 72 h and the dry biomass of each beetle was determined with a precision of 0.001 g.

Figure 9.3 - Measured body traits on *Geotrupes spiniger* (Marsh. 1802) with HL: head length, HW: head width, PL: pronotum length, PW: pronotum width, PH: pronotum height, EL: elytron length, pTL: protibia length, pTW: protibia width and mTL: metatibia length. ©www.istockphoto.com

Data analysis

Non-metric multidimensional scaling (NMDS) ordination techniques were used to examine whether dung beetle taxonomic composition differed between grazing regimes. NMDS analyses were performed using the function *metaMDS* from the R

package 'vegan, version 2.3-5' (Oksanen et al., 2016) which uses a Bray-Curtis dissimilarity matrix of the species abundance data as a default. The *envfit* function (vegan package) with 1000 randomization tests was used to evaluate differences in dung beetle assemblages between grazing regimes. Sampling completeness was assessed, using species accumulation curves and by estimating the number of missing species. Species accumulation curves were calculated with the *specaccum* function (vegan package) with 100 random permutations as a measure of sampling completeness in each study site. The number of missing species was estimated by Chao bias-corrected, first-order jackknife, second-order jackknife and bootstrap methods using the *specpool* function (vegan package). Dung beetle communities were furthermore defined by species diversity (number of species), total abundance, Shannon-Weaver diversity index (H') and evenness (J) (Hill, 1973; Jost, 2006) by the using the following formulae:

$$H' = -\sum_{i=1}^{S} p_i \ln (p_i)$$

$$J = \frac{H'}{\ln (S)}$$

where p_i is the proportion of individuals belonging to the i^{th} species in the dataset of interest and S is species richness.

Two-sample t-tests were used to compare species abundance by study site. Furthermore, total dung beetle biomass was calculated for each site by multiplying and summating species abundance and specific biomass measured on individuals sampled at that study site. For species sampled at both sites, morphological metrics and individual biomass were compared between grazing regimes using one-way ANOVAs. Furthermore, dung beetle species were assigned to functional groups and size classes (Doube, 1990; Slade et al., 2007). For each functional group and size class, total dung beetle biomass was calculated per study site and Kruskal-Wallis tests were used to determine significant differences between sites, and hence between grazing regimes.

Dung removal was calculated as follows:

$$M_{removed} = \frac{M_{reference} - M_{sample}}{M_{reference}} \; x \; 100$$

where $M_{reference}$ is the average dry mass of 300g of fresh dung and M_{sample} is the dry mass of the remaining dung at the end of the experiment. Dung removal was arcsine square-root transformed in order to meet the assumptions for parametric testing and in a two-way ANOVA the effects of grazing regimes and treatment units were evaluated. All statistical analyses were run in R version 3.3.1. (R Core Team, 2016b).

Results and discussion

Dung beetle diversity

During the 48h sampling period, more species (9 versus 4 species) and higher dung beetle abundance (852 versus 164 individuals) were caught in the extensively grazed grassland which confirms the findings of lower dung fauna diversity in intensive agricultural systems (e.g., Hutton and Giller (2003), Lobo et al. (2006), Tonelli et al. (2017)) (Table 9.4).

Table 9.4 - List of sampled dung beetle species and the total number of individuals by grazing regime. Asterisks indicate significant differences between study sites after applying two sample *t*-tests with ***: $p<0.001$, **: $0.001<p<0.010$, *: $p<0.050$.

		grazing regime	
sampled species	**functional group**	**extensive**	**intensive**
Geotrupidae			
Geotrupes spiniger (Marsham, 1802)	paracoprid	16	60**
Scarabaeidae			
Onthophagus coenobita (Herbst, 1783)	paracoprid	20	3*
Onthophagus similis (Scriba, 1790)	paracoprid	124	0**
Acrossus rufipes (Linnaeus , 1758)	endocoprid	661	93**
Agrilinus ater (De Geer, 1774)	endocoprid	1	0
Aphodius foetens (Fabricius, 1787)	endocoprid	6	0**
Bodilopsis rufa (Moll, 1782)	endocoprid	12	0**
Nimbus contaminatus (Herbst, 1783)	endocoprid	9	0
Teuchestes fossor (Linnaeus, 1758)	endocoprid	3	8
biodiversity indicators	total number	852	164
	total biomass (g)	25.6	10.8
	species richness	9	4
	Shannon-Weaver index	0.8	0.9
	evenness	0.4	0.7
	chao ± SE	9.0±0.4	4.0±0.0
	jack1 ± SE	9.9±0.9	4.0±0.0
	jack2	10.7	3.3
	boot ± SE	9.4±0.6	4.1±0.3

In absolute numbers, both sites were dominated by dwellers which is in line with the expectations for north temperate European dung beetle communities which are generally dominated by dweller species accompanied by some tunnelling species (Hanski and Cambefort, 1991b). Furthermore, the species list was comparable with earlier results for the Zwin area in late summer (Milotić et al., 2017), although dung beetle community composition differed significantly between grazing regimes (R^2= 0.5676, p= 0.001) (Figure 9.4). Of the species that were sampled at both study sites, *Onthophagus coenobita* and *Acrossus rufipes* were significantly more abundant under extensive grazing, while the opposite was found for *Geotrupes spiniger* which was collected almost 4 times more often at the intensively grazed site (Table 9.4).

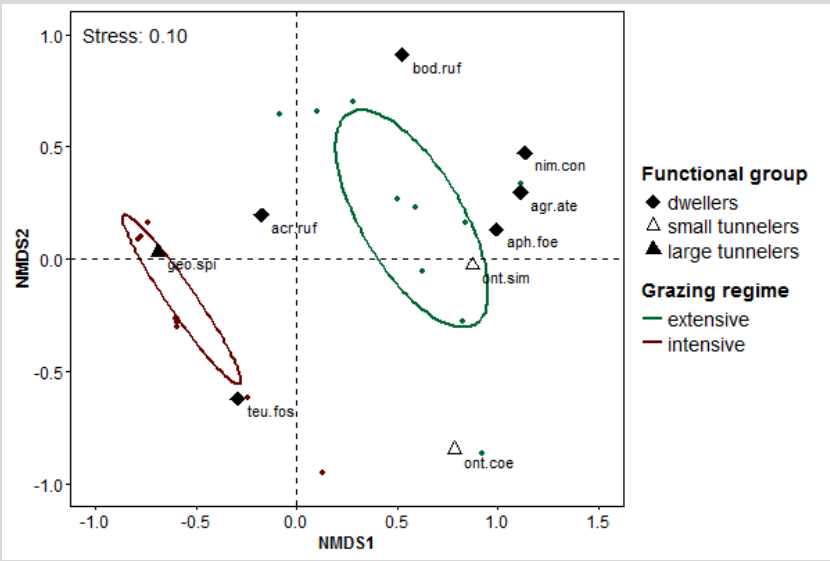

Figure 9.4 - Non-metric multidimensional scaling ordination of dung beetle community structure by grazing regime. Red and green dots represent sampling locations in the intensively and extensively grazed sites respectively. Species codes are acr. ruf, *Acrossus rufipes*; agr.ate, *Agrilinus ater*; aph.foe, *Aphodius foetens*; bod.ruf, *Bodilopsis rufa*; geo.spi, *Geotrupes spiniger*; nim.con, *Nimbus contaminatus*; ont.coe, *Onthophagus coenobita*; ont.sim, *Onthophagus similis* and teu.fos, *Teuchestes fossor*.

Dung beetle morphological traits

In terms of biomass, the intensively grazed Hazegras pasture was dominated by large paracoprids while in the Zwin area endocoprids were the predominant functional group in terms of absolute numbers, diversity and biomass. *Geotrupes spiniger* was the sole large tunneler sampled during this experiment and accounted for 77% and 27% of the total dung beetle biomass under intensive and extensive grazing respectively. This species is one of the most common *Geotrupes* species in Belgium and has a distinct preference for cattle dung (Janssens, 1960; Wassmer, 1995). Furthermore, the individual beetle mass was significantly higher at the extensively grazed site for the tunnelling species *Geotrupes spiniger* and *Onthophagus coenobita* (Table 9.4).

Significantly higher values were found under extensive grazing for elytron length, head width, pronotum height, pronotum length, pronotum width, protibia length, protibia width and metatibia length of *Geotrupes spiniger* and for pronotum width and protibia length of *Onthophagus coenobita* (Figure 9.5).

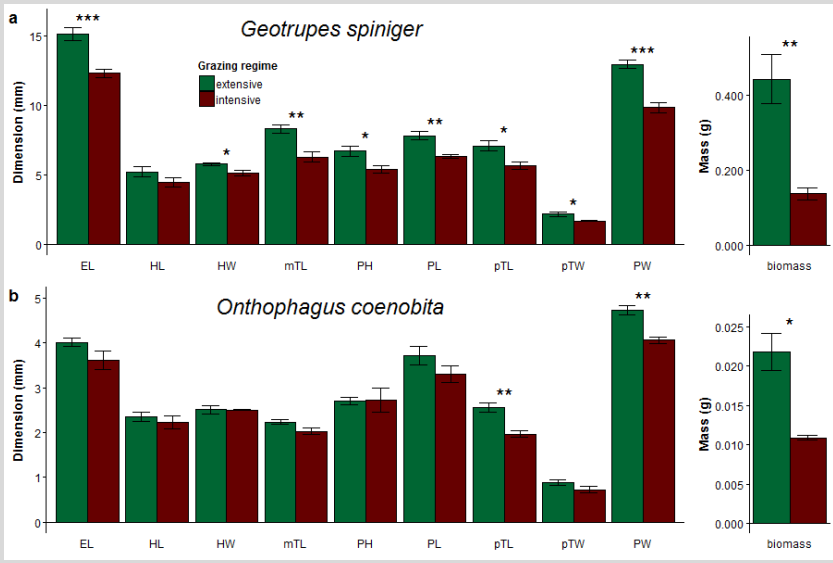

Figure 9.5 - Morphological metrics and biomass of *Geotrupes spiniger* (a) and *Onthophagus coenobita* (b) by grazing regime. In case metrics differ by site after applying one-way ANOVAs the resulting p-values are plotted as asterisks with ***: $p<0.001$; **: $0.001<p<0.010$ and *: $0.010<p<0.050$. Measured body traits are EL: elytron length, HL: head length, HW: head width, mTL: metatibia length, PH: pronotum height, PL: pronotum length, pTL: protibia length, pTW: protibia width and PW: pronotum width.

More dung beetle biomass was sampled at the extensively grazed site, with a significantly higher biomass of small paracoprids (*Onthophagus coenobita* and *Onthophagus similis*) and endocoprid species (Figure 9.6). Although the cattle at the intensively grazed site did not receive any veterinary treatment in the two months preceding the experiment, the temporal usage of veterinary parasiticides might have a long lasting impact on dung invertebrate populations (Wardhaugh, 2005). Depending on the nature, timing and frequency of the treatment, the effect of drug residues in cattle dung may range from negligible to catastrophic for dung beetle populations (Wardhaugh et al., 1998). Anthelmintics such as ivermectin may result in increased mortality of newly emerged dung beetles and delayed sexual maturation (Wardhaugh et al., 2001). Although a literature search did not reveal a direct link between dung beetle morphology and the use of veterinary products, smaller adult beetles could be a non-lethal effect of parasiticides. Strong (1992) reported that the adult emergence of several Diptera species was reduced and the imagos showed an increased number of morphological abnormalities due to the use of ivermectin. Recently, González-Tokman et al. (2017) found a positive relation between the use of agricultural herbicides and the mature body size of the dung beetle *Euoniticellus intermedius* which suggests that pollution with chemical products could have various effects on dung beetle populations.

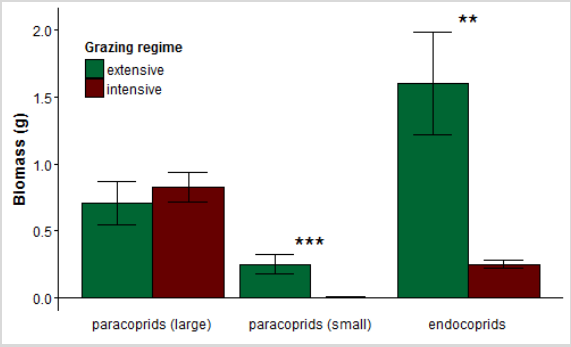

Figure 9.6 - Average biomass per sampling pot, classified according to functional groups. Significance levels of significantly differing grazing intensities are indicated as follows: ***: $p<0.001$; **: $0.001<p<0.010$ and *: $0.010<p<0.050$.

Dung removal

Dung removal was more than 2 times higher in the intensively grazed site (Figure 9.7 and Table 9.5) which is contradictory to the theory that the less diverse dung beetle assemblages found under high grazing pressure results in a loss of ecosystem functioning such as dung decomposition (Hutton and Giller, 2003; Negro et al., 2011). However, as in none of the study sites did dung removal differ between control and experimental treatments, the role of dung beetles in dung removal appears to be rather limited in the short time frame of the experiment (48h). Furthermore, in line with the recommendations of the protocol, dung originating from the same batch was used in both areas. The fact that the structure of dung is highly correlated with herbivore diet (Holter, 2016) could also affect dung beetle behaviour. In both Belgian study sites, dung from temporarily stabled cattle from the Hazegras site was used. As dung from cattle fed on silage generally is more fluid compared to dung from free-ranging cattle (Aschenborn et al., 1989), dung beetles at the Zwin site might have preferred dung from the resident herbivores over the experimental dung pats.

Table 9.5 - Results of the two-way ANOVA using dung removal as measurement variable and grazing regime, treatments and their interaction term as fixed effects.

factor	*df*	*F* value	*p*
grazing regime	1	6.068	0.021
treatment	1	2.218	0.148
grazing regime x treatment	1	1.132	0.297

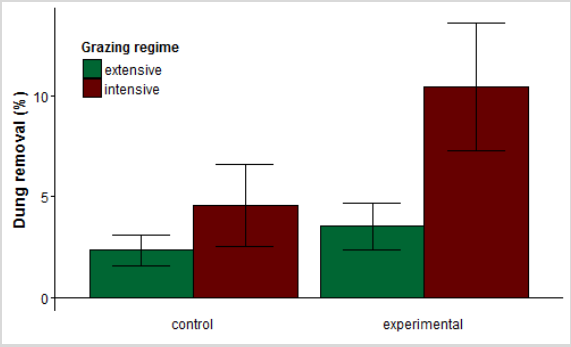

Figure 9.7 - Dung removal in control and experimental units by grazing regime. Full ANOVA results can be found in Table 9.5.

Conclusion

Clear differences exist in dung beetle assemblage structure between grazing management types. Although the initial higher dung removal rates at the intensively grazed grassland are beneficial for pasture quality, the smaller size of the beetles in this area is rather surprising. Furthermore, the higher dung beetle diversity under low grazing pressure stresses the importance of the use of low numbers of large herbivores in nature management. Whether the often highly significant differences in dung beetle assemblage composition, dung beetle abundance and dung beetle morphology in this local study will be confirmed within the much larger framework of the worldwide experiment, of which all results are not yet available, is subject to further analyses. In this large-scale experiment, geographically diverse replicates will be included, which should allow strong underpinned results, in which site effects are better dealt with than in the present local case study.

Acknowledgments

We thank the Agency for Nature and Forest (ANB, Flemish government) and the forester Koen Marechal for access to the Zwindunes and Polders nature reserve and Gerard Stroo for granting access to his pasture and logistical help.

9.3.2 Dung removal and secondary seed dispersal

Qualitative aspects of dung

Although dung beetles feed on dung both in the larval and adult phase, the structural and chemical composition of the consumed dung is different between life stages. Whereas adult beetles are filter-feeders which typically feed on the liquid content of fresh dung, larvae are bulk feeders and feed on older dung in pats or in underground brood masses (Holter, 2016). As the liquid fraction of dung quickly evaporates after deposition (Holter and Scholtz, 2007) and dung beetles use their olfactory senses to locate dung (Dormont et al., 2004; Dormont et al., 2007), adult dung beetles have a strong preference for fresh dung which is less than a week, and usually only a few days or even hours old (Finn and Giller, 2002; Holter and Scholtz, 2007). Although the ecology of the beetles, climate factors and the presence of other insects inside the dung pat may affect the selection of dung resources by adult beetles (Dormont et al., 2010; Holter, 2016), the general patterns in resource selection were confirmed in this study as most species and individuals were sampled within 1 week (chapter 7 and 8) or even 48 h (box 9.1) after the deposition of fresh dung.

Furthermore, most dung beetle species prefer a certain type of dung. Whereas habitat and resource specialists are closely linked with certain habitats and dung types, more plasticity is found in generalist species which adapt their feeding preferences according to the availability of resources (Bourg et al., 2016). Most species prefer dung from either hindgut fermenters (e.g., horses) or ruminants (e.g., cattle, sheep, deer and goats) (Mroczyński and Komosiński, 2014), although this effect was not always straightforward when comparing different seasons and study sites in the multi-site experiments (chapters 7 and 8). The selection for a certain dung type is defined by competitive interactions with other dung-inhabiting species and the nutritive content of the faecal depositions (Bogoni and Hernández, 2014). Furthermore, the dung structure could explain the preference for dung from either ruminants or hindgut fermenters. Whereas the dung from hindgut fermenters mostly consists of light, uncompressed dung that still contains large plant particles, ruminant dung has a smaller average particle size and is much denser (Steuer et al., 2013). Furthermore, the size of ingested dung particles is positively related with dung beetle size and differs between functional groups with rollers accepting larger particles

than tunnelers of comparable size (Holter and Scholtz, 2005). Consequently, the preference for a certain dung type and, hence, dung decomposition rate, may be closely related to the composition of local dung beetle assemblages. Another point of interest is the origin of dung. The structure of dung is highly correlated with herbivore diet (Holter, 2016), for example, cattle fed on silage produce more fluid dung compared to free-ranging cattle (Aschenborn et al., 1989) which soon forms a hard and dry crust. This structural variation in dung sources may affect dung beetle behaviour as the hard top layer might restrict tunnelling. This problem was overcome by using field-collected dung in chapters 7 and 8, but due to practical considerations, dung from temporarily stabled cattle was used in the experiment of box 9.1. As in this experiment, dung originating from an intensively grazed site was also used in an extensively managed grassland, dung beetles at the latter site might have preferred dung from resident Galloway cattle over the experimental, 'alien' dung pats.

Consequences of functional diversity for dung removal

In the multi-site experiments (chapters 7 and 8), dung removal rate was related to the functional composition of dung beetle assemblages and species body size. Evidently tunnelers, and rollers when present, removed more dung compared to dwellers which coincides with the conclusions of Nervo et al. (2014) in the Italian Alps and Slade et al. (2007) in Malaysian tropical forests. Furthermore, body size was a major determinant of dung decomposition as large tunnelling beetles were far more efficient dung removers than small tunnelers which confirms the earlier findings of Kaartinen et al. (2013) in North Europe and Andresen (2002a) and Slade et al. (2007) in South American and Asian tropical forests. The varying efficiency in dung removal between functional groups can be attributed to differing nesting strategies. Adult dwellers feed on the liquid fraction of dung and their larvae feed on the more fibrous part of dung in the dung pat itself or directly underneath it. Even within this group, a further distinction can be made according to the specific nesting locations with soil ovipositing dwellers removing more dung at a faster pace than dung ovipositing dwellers (Manning et al., 2016). In contrast, tunnelers remove more dung on a short term as they supply their underground burrows with brood balls soon after the deposition of fresh dung. In a field mesocosm experiment,

Nervo et al. (2014) found significant effects of dweller assemblages after one year whereas less than one month was needed to find an effect of tunnelling species. Although mineralisation ultimately does not differ between dwellers and tunnelers (Nervo et al., 2017), the rate at which dung disappears is relevant as well, specifically in combination with agro-pastoral activities. Due to the strong odour and plant lignifications, the surrounding area of dung pats is avoided by grazing livestock (Haynes and Williams, 1993). Thus, if dung is buried quickly after deposition a larger pasture area remains suitable for grazing (Miranda et al., 2000).

Although dung beetles are major dung decomposers in pasture ecosystems, other soil-inhabiting macro-invertebrates were performing a similar role in ecosystem functioning, especially in the northern study sites in the multi-site experiments (chapter 7 and 8). Earthworms are known to incorporate faeces into the soil and alter the nitrogen availability of soil through complex microbial interactions in their gastrointestinal tract (Gittings et al., 1994; Groffman et al., 2004). In the cool, wet climate of Scandinavia earthworms may even remove the entire dung pat before dweller larvae have completed their development (Gittings and Giller, 1999). Although separate functional groups of dung beetles and macro-invertebrates may move considerable amounts of dung, combinations of functional groups resulted in more dung removal in the multi-site experiment in the Western Palaearctic. This confirms the results of earlier research on a smaller scale (e.g., Manning et al. (2016), Nervo et al. (2014), Nervo et al. (2017)) and stresses the need to conserve and protect complete dung beetle assemblages in order to warrant the maintenance of ecosystem functioning related to dung removal. Conservation measures could include the protection of pasture areas with a long grazing history, the reinstatement of agropastoral activities in abandoned areas, the protection of coastal areas which still contain roller species and the judicious use of anthelmintics and other veterinary drugs or the development of other, less destructive formulations.

Secondary seed dispersal by dung beetles

Dung beetles are able to move vast amounts of dung which may have a major impact on the fate of seeds embedded in dung. Recent research in tropical biomes has revealed that dung beetles do not treat dung with seeds

differently than seed-free dung and move seeds rather accidentally while handling dung (Braga et al., 2013; Slade et al., 2007). Hence, dung beetles may facilitate seed survival by increasing the probability that seeds will escape predation by rodents and germinate (Andresen, 2003; Andresen and Levey, 2004; Beaune et al., 2012; Shepherd and Chapman, 1998).

Given the combination of the nearly global distribution of dung beetles in various habitats (from pristine to anthropogenic environments) and the frequent occurrence of endozoochory, secondary seed dispersal by dung beetles is thought to be a very widespread phenomenon (Vander Wall et al., 2005). Nevertheless, many factors have been associated with the probability of secondary seed dispersal and its impact on the fate of the dispersed seeds. As dung beetle species often prefer certain food resources, the dung type in which the seeds are embedded will largely define the probability of secondary seed dispersal (Santos-Heredia et al., 2011). Dung beetle assemblage compositions may differ between habitats and seasons which may have a likewise effect on seed relocation patterns (Andresen, 2003; Petre et al., 2015). Furthermore, the size of seeds plays a role with smaller seeds being more likely to be dispersed than larger ones (Andresen and Feer, 2005; Andresen and Levey, 2004; Slade et al., 2007). In general, the number of buried seeds increases with dung pat size (Andresen and Levey, 2004) and dung beetle size (Andresen and Feer, 2005), although this correlation might change to a negative relationship in case the dung contains very high seed densities (Shepherd and Chapman, 1998).

In the multi-site experiments in chapters 7 and 8, the disappearance of seeds was indeed positively related to dung removal although smaller seeds were dispersed more often. Furthermore, an effect of functional composition was found as functionally more diverse assemblages removed more seeds. These experimental results from the Western Palaearctic zone are in line with the conclusions of earlier research in tropical biomes (e.g., Slade et al. (2007)) and stress the relevance of functional completeness. Nevertheless, seed removal differed among functional groups with tunnelers being the most important seed dispersers while dwellers barely had an effect. The difference in seed dispersal effectiveness lies in the nesting behaviour of dung beetles. Dwellers oviposit inside the dung deposition or directly underneath it, while rollers and tunnelers actively move dung particles in vertical and horizontal directions. From the plant's perspective, the depth at which seeds are buried is crucial in determining

the fate of seeds. Whereas rollers eventually bury their dung balls at a shallow depth, the tunnel depth is positively correlated with body size in tunnelling species (Gregory et al., 2015). As germination requirements differ between plant species and are related to burial depth (Limón and Peco, 2016), burial by dung beetles may negatively affect germination. In a temperate grassland, D'hondt et al. (2008) found a negative impact of the presence of large tunnelers on seedling recruitment of coastal grassland plant species, whereas other studies found the opposite effect when functionally complete dung beetle assemblages were present (e.g., in Amazonian tropical forests (Andresen and Levey, 2004)).

Implications for post-dispersal seed fate

Although dung beetles may even play a minor role in primary seed dispersal, for example through the dispersal of the strongly scented seeds of *Ceratocaryum argenteum* in South Africa (Midgley et al., 2015), secondary seed dispersal by dung beetles may have a larger impact on the composition of plant communities than primary dispersal by endozoochory (Chambers and MacMahon, 1994; Vander Wall and Longland, 2004). The directed distribution to micro-sites suitable for germination and the reduction of competition with other seedlings may have positive effects on the establishment and fitness of plants (Andresen, 2001). In alpine meadows, Nervo et al. (2017) measured an increased cover of mesotrophic species due to the dung decomposing actions of dung beetles and the subsequent release of mineral nitrogen compounds, whereas the cover of oligotrophic species was left unchanged. Although interactions between species and functional groups of species enhance many ecosystem functions, tunnelers particularly affect dung decomposition due to the large amount of dung removed by this group (see chapters 7 and 8, and Manning et al. (2016), Nervo et al. (2017), Slade et al. (2007)).

As the physical fragmentation of faeces facilitates seedling emergence and the establishment of faeces-embedded seeds (Faust et al., 2011; Mancilla-Leytón et al., 2012), changes in the structural composition of dung pats due to dung beetle activity may positively affect the post-dispersal fate of seeds. While moving through dung, beetles may break large dung depositions into smaller pieces and bring seeds to the surface layers. Also, the digging

behaviour of tunnelers may crack the hard crust that often appears on fluid ruminant dung and seedlings may make use of the tunnel holes. Due to the fact that adult dung beetles are filter feeders which feed on the liquid fraction of dung and leave the coarse particles as a loose heap of garbage (Holter, 2016), no seeds are consumed while feeding. During this research, a visual observation was made of how a mass occurrence of small dwellers (mainly *Melinopterus prodromus*) broke the initially firm structure of horse dung into a heap of small dung particles in a grassland area in Ruiselede, Belgium. Besides the fact that such actions result in an increased decomposition rate, endozoochorous seeds may benefit from enhanced germination conditions in the remains of this dung pile. On the other hand, the burial of seeds in brood balls intended for feeding tunneler or dweller larvae may provide a nutritive environment for establishing seedlings. In a field experiment in an Amazonian forest, Santos-Heredia et al. (2016) measured a higher foliar phosphorus concentration in seedlings of native trees caused by secondary dispersal by dung beetles. The brood balls may, therefore, supply small amounts of organic fertiliser which increase seedling growth rates and enhance the establishment opportunities of secondarily dispersed seeds compared with undispersed or wind-dispersed seeds (Andresen, 1999; Andresen, 2002a; Estrada and Coates-Estrada, 1991; Shepherd and Chapman, 1998). Furthermore, secondary seed dispersal may reduce competition as seeds are transported in smaller quantities toward underground burrows. Although the tunnels of some tunneler species may be too deep to allow germination (D'hondt et al., 2008), the removal of seeds may also have an impact on the seeds remaining in the dung pat due to the decrease of the scramble competition for space and nutrients by seedlings (Andresen, 1999; Andresen and Feer, 2005; Andresen and Levey, 2004; Nichols et al., 2008).

9.4 Perspectives for future research

As mentioned earlier, the post-dispersal fate of seeds largely depends on the complex interplay of many factors such as the effects of gastrointestinal passage on the further development of seeds, the quality of the deposition site (e.g., dung type, but also habitat type and the vegetation underneath dung depositions), the seed content of the dung pat and the competitive interactions between emerging seedlings, secondary seed dispersal by coprophagous macro-invertebrates and post-dispersal seed predation. In this thesis, the impact of a selection of these factors has been studied in relation to the establishment success of a limited set of grassland species. From these experimentally obtained results, a qualitative cost-benefit estimation has been deduced. Due to practical considerations and for the sake of clarity, we chose to study the impact of each factor separately. For example, when studying the germination of seeds in dung, intact and thus uningested seeds were used. As the digestive processes may have an effect on the germinability and germination timing of seeds, the outcome of the experiments may differ if ingested seeds had been used. Future studies could use the methods of Bruun et al. (2008) and Yamashiro and Yamashiro (2006) to retrieve ingested seeds from dung in order to test their germinability, establishment and growth in dung and in competition with other species. In the laboratory experiments in the first section of this thesis (chapters 3 to 6), dung from stabled cattle was used in order to prevent contamination with wild seeds. Although we were able to rule out interference with wild seeds, dung from cattle fed on silage is more fluid after deposition and soon develops a very hard crust which is hard to penetrate. This hard crust might have negatively affected germination in our experiments. As dung from free-ranging animals is more fibrous, we might expect to find more germination on field. Therefore, it could be useful to estimate germination metrics in dung from both free-ranging and stabled animals in future experiments.

Furthermore, species differed in their response to the experimental treatments. Expanding the set of species might provide more clarity in the endozoochorous dispersibility of plant species. Although the species used in the experiments have been previously found in endozoochory studies, the cost - benefit ratio might differ between species. As suggested by Carmona et al. (2013), a further classification of species into grazing increaser and

grazing decreaser species while selecting species for experimentations might give us more insight into the overall consequences of endozoochory. Another problem in the calculation of a cost - benefit ratio for endozoochorous dispersal is the different nature of the costs (mainly time, opportunity and risk costs). Although it is possible to compare the costs of the outcome of each step in the endozoochorous dispersal process with the costs of unassisted seeds, it remains difficult to accurately calculate the overall quantitative costs. There is a need to define some kind of universal 'currency' for adding up the costs and benefits for the entire dispersal process of plants.

Although the dispersal of seeds over a relatively short distance has clear consequences for vegetation composition and conserves the genetic diversity of plant populations, long distance dispersal events may have had more profound effects on the current distribution of plants. In the light of the future challenges of a changing world due to climate change, increasing urbanisation and fragmentation, and the increasing distribution of invasive species, a better understanding of these rare events might be useful in policy making and predictive modelling. Future research could, therefore, use a backward approach as suggested by Wang and Smith (2002) and infer seed dispersal patterns from the current distribution of species. Genetic techniques and ecological models might be useful to map past dispersal processes in order to make predictive models for future dispersal patterns. Another method could be to work forward by labelling and tracking large amounts of diaspores and seed dispersers in order to estimate the probability for long distance dispersal as proposed by Nathan (2006).

Another point of interest is the impact of different combinations of herbivore species. In many places in the world, herbivore species have gone extinct and with them, the dispersal opportunities for certain plants species may have vanished. As shown in previous studies and in this one, herbivores may differ in seed dispersal effectiveness. A combination of different domestic herbivore species or domestic herbivores with native wild herbivores might be more useful for seed dispersal than a monospecific herbivore fauna. The plans of the European Rewilding Network to reintroduce European bison, wild horses and/or the back-bred bovine relatives of the aurochs in large connected areas of their ancestral habitats could restore some of the ancient seed dispersal mechanisms and patterns. Furthermore, in areas with a high level of fragmentation such as Western

Europe, climate change may have profound effects on future vegetation patterns. In this regard, plants have three options and should either adapt to the changing environment, tolerate moderate environmental changes without major genotype changes, or disperse towards habitats which have become suitable for colonisation. A changing climate might also result in more migration of wild herbivores and as such herbivores can be considered as seed reservoirs, endozoochory might be an important dispersal mechanism for the migration of plant communities and the maintenance of genetic diversity in plant populations.

Besides the effects of primary dispersal, global change may also have a large impact on secondary seed dispersal patterns. As the functional distribution of dung beetles is largely defined by the current minimum temperatures, an increase of the lowest temperature can be expected to result in the northward migration of certain roller and tunneler species which could have a profound effect on future dung removal and secondary seed dispersal patterns. Experimental manipulations of local dung beetle assemblages (e.g., through the introduction of new species in a mesocosm environment) could provide further knowledge of how changing dung beetle assemblages affect ecosystem functioning. Another challenge regarding dung beetle diversity lies in the effects of habitat deterioration (e.g., land use change, loss of connectivity of suitable habitat patches) and the quantity and quality of their food resources. In certain areas, the quantity of dung may be a bottleneck for dung beetle diversity due to the decrease in dung supply of wild herbivores caused by hunting and habitat loss, or in the supply of domestic herbivore dung caused by the cessation of agropastoral practices. In areas with a high density of agro-industrial businesses, the expectedly poor quality of dung resources might restrict dung beetle abundance and richness due to the poor structural quality of dung from animals on an artificial diet and the presence of drug residues in dung. Although many studies have already proven the adverse effects of veterinary drugs on coprophagous macro-invertebrates, many questions still remain unanswered. Future research could therefore further look into the effects of pharmaceutical residues on the morphometric traits of dung beetles and functional diversity and the relation with the ecosystem functions provided by this insect group.

10 Conclusions

The initial steps in the endozoochorous dispersal process encompass a large cost for many temperate grassland species. Due to the specific mechanical, thermal and chemical actions of the digestive system of large herbivores, seeds are often less likely to germinate after egestion, which eventually results in a tremendous loss of once viable seeds in many grassland species. If they leave the animal body in a viable condition, all survival troubles are not yet overcome.

In general, due to the specific conditions in the digestive system seeds need more time to germinate compared with undigested seeds. Secondly, after the dispersal process has been successfully passed, a further filtering is accomplished at the deposition site. The specific environmental conditions in the dung pat inhibit the germination and establishment of seedlings which results in a further seed loss and a delay of germination. However, in the later developmental phases, the nutritive compounds in the dung pat become useful as established seedlings grow faster and are able to invest more in reproductive tissues. The initial losses of viable seeds in the digestive system and in the grim environment of the dung pat therefore seem to be compensated up to a certain extent in enhanced growth and flowering of the survivors.

Furthermore, dung pats, being spatially isolated habitats with very specific conditions, may contain high seed concentrations which may trigger processes of inter- and intraspecific competition. In an attempt to win the competition, certain plant species may produce more biomass and flowers. The specific environment of dung not only delays the establishment of seedlings but may enhance competition in the later developmental phases. Herbivore grazing may be a trigger for the expansion of some plant species.

In this thesis, the causes of the increased distribution of *Helianthemum nummularium* after the introduction of large ungulates were studied. Although the selective grazing behaviour with diet preference for grasses most probably creates an appropriate and sufficiently open mosaic grassland-scrub environment for plant establishment and maintenance, proof was also found for the zoochorous dispersal of this species. The

hooves and fur of cattle and horses are fit for the epizoochorous transport of seeds, while seeds were able to survive the digestive tract reasonably well and establish in dung (endozoochory).

The post-dispersal fate of seeds is closely linked with the fate of dung, and its decomposition rate. Although dung decomposition is affected by many factors, the activity of dung beetles is particularly interesting regarding endozoochory. Dung beetles are excellent secondary seed dispersers as they move considerable amounts of dung and accidentally bury the endozoochorous seeds present in dung particles. In an experiment conducted at two study sites in Belgium, dung beetles removed vast amounts of dung and seeds. Nevertheless, a great difference was found between functional groups with large tunnelers removing most dung and seeds. Similar patterns were found on a larger, continental scale. Although the composition of dung beetle assemblages differed along a north-south and east-west gradient and thus between climate and biogeographic zones, dung removal varied with functional group composition.

When present, rollers and large tunnelers removed most dung and seeds, while dwellers were least effective. Other soil inhabiting macro-invertebrates, such as earthworms, partially took over the dung removing actions in the absence of tunnelers. Furthermore, dung removal was positively correlated with the functional completeness of dung beetle assemblages. This stresses the need of functional group conservation in order to maintain ecosystem functions of dung removal and the herewith linked secondary seed dispersal.

11 Summary

Dispersal options of sessile organisms such as plants are rather limited. Unlike most animals which can move actively between habitat patches by choice, plants are mainly restricted to passive dispersal processes. The most common dispersal structures of plants are seeds or fruits, although some plant species have vegetative structures which are specialised for dispersal (such as rhizomes, bulbils or even the entire plant). Hence, in the case of seed dispersal, the dispersal process can be seen as the mobile link between the reproductive cycle of the maternal plant and the start of a new life cycle by the establishment of its offspring. Despite the restriction to passive dispersal, seed dispersal methods are rather diverse and include the use of wind, water, gravity or animals as dispersal vectors. The free-ranging domestic herbivores, such as cattle, horses and sheep, which have been commonly introduced as a nature management measure in temperate grasslands, are known to transport massive amounts of seeds through ingestion and subsequent defecation of viable seeds with dung (*endozoochory*) or through attachment to their fur or hooves (*epizoochory*). Although the main reason for the introduction of these ungulates is to create spatial heterogeneity and to prevent shrub encroachment, the dispersal of plant seeds is a side effect with a profound effect on vegetation structure and dynamics. Particularly endozoochory is a complex process including multiple steps of seed uptake, seed transfer, the deposition with dung, and all the subsequent processes of germination, establishment, growth and maturation in the post-dispersal environment. In recent decades, extensive proof for the existence and relevance of endozoochorous dispersal in semi-natural landscapes has been provided through germination trials with field collected dung. As such, these studies have provided a valuable insight in the dispersal probability of a wide array of grassland species, but few of them have examined the consequences of the qualitative aspects of endozoochorous dispersal. Nevertheless, the quality of the seed-dispersing herbivore and the post-dispersal environment may have a decisive impact on seed fate.

In **chapter 2** the different aspects of the transfer of seeds through the gastrointestinal tract of herbivores were studied. The germination of grassland plants was evaluated after simulated herbivore digestion in a

standardised laboratory environment. Ruminants (cattle) and hindgut fermenters (horses) were used as model organisms in the experiments. Three major digestive processes were studied through mechanical, thermal and chemical treatments of the seeds simulating mastication, body temperature and digestive fluids, respectively. A selection of 15 relatively common grassland species was made including congeneric groups of annuals and perennials of Cistaceae, Cyperaceae, Fabaceae, Poaceae and Urticaceae species. The simulated gastrointestinal environment of cattle and horses had a comparable effect on germination, and major differences in germination behaviour were found among plant species. For most of the tested plant species, treatments had a decelerating and inhibiting effect on germination compared to the untreated seeds. However, species of the Cistaceae and Fabaceae benefitted from mechanical treatments. Thermal treatments, simulating the body temperature, inhibited germination in most cases. While certain species inevitably paid a cost for dispersal through the loss of germinable seeds, species of the Cyperaceae and Poaceae were hardly impaired by any of the treatments and even germinated better after chemical treatments. Furthermore, the germination success of *Urtica urens* was even higher after each of the treatments, which suggests seeds are specifically well adapted to gastrointestinal passage, and hence to endozoochorous dispersal.

Through the differential uptake of seeds and the loss of seeds of certain species during digestion, ungulates act as an ecological filter in the first step of the endozoochorous dispersal cycle. In the following steps of germination, establishment and growth, environmental factors determine the fate of dispersed seeds. The nutritive content and the specific structure of dung depositions may act as a second filter for endozoochorously dispersed seeds. In **chapter 3** the germination probability and timing of 15 annual and perennial temperate grassland species was experimentally assessed in the dung of cattle and horses. Germinability and germination timing largely differed between species, but in all tested species the presence of dung resulted in much longer germination times and lower germinability. These adverse effects were stronger if cattle dung was used, which could be related to its structural composition. Although horse dung was more compacted than cattle dung at the moment of deposition, a hard crust appeared on cattle dung soon after deposition. Furthermore, the higher nutrient content found in cattle dung might be irrelevant in the

germination phase as young seedlings rely on the reserve of mineral and organic nutrients in the seed, and most of the nutrients present in fresh dung pats are unavailable for plant uptake. The more fibrous and loose structure of horse dung was, therefore, less restrictive for the germination and early establishment of seedlings compared with the hard crust on cattle dung that hinders the penetration of young seedlings.

However, once germinated and established as a young seedling, seedlings may increasingly benefit from the nutrients leaching from the decomposing dung pat. In an attempt to quantify the impact of the dung environment on the later stages of plant development, seedling biomass and growth rate, height, ramification, flowering and biomass of grown plants were measured (**chapter 4**). In the early developmental phase, dung did not seem to have an impact as seedling biomass did not differ between dung and dung-free control environments. However, in the later growth phases, most of the tested species benefitted from being deposited in dung, which was measured with higher growth rates, more reproductive output and higher biomass of mature plants. As a consequence, the initial losses of viable seeds through the digestive system of herbivores and through the reduced germination in dung were partially compensated by enhanced growth and flowering in some species.

Depending on the herbivore species and season, dung pats may contain a large assemblage of conspecific or heterospecific seeds competing for space, light and nutrients in the space-limited environment of an individual dung pat. In an environment rich in nutrients, such as herbivore dung, the outcome of inter- and intraspecific competition might differ from situations where nutrients are limiting. Additionally, the typical temporary island character of dung pats may impact competitive interactions by the spatial concentration of seeds that were dispersed endozoochorously. It can hence be expected that complex interactions exist between different seed densities, species combinations and the quality and structure of the dung pat. In **chapter 5**, the effects of competition between three common perennial grassland species were studied in a greenhouse experiment. *Agrostis stolonifera*, *Trifolium pratense* and *Trifolium repens* were used in two-species combinations with different proportions of each species and in monocultures. Seeds were sown in three seed densities and the effects of cattle and horse dung on establishment, growth and flowering were measured. Interactions differed between species mixtures and could be

inferred to interspecific competition. Seeds sown in polycultures generally germinated faster, but the resulting seedlings had lower relative growth rates. The presence of competing species led to an increase in the biomass of species growing in polycultures while intraspecific competition was found in monocultures. Relatively more flowers developed when plants were growing in polycultures. Few effects of seed densities were found, although higher seed densities led to lower establishment success in both monocultures and polycultures. As in the previous experiments the addition of dung increased the time to first germination and relative growth rates, but decreased establishment success in monocultures. Compared to horse dung, cattle dung gave rise to more intense interspecific competition and resulted in a lower seedling establishment, lower biomass and more flowers. As a result, the post-dispersal fate of seeds is shaped by both seed density and the presence of dung. While high seed densities imply a cost due to seed loss, the nutritive environment of dung acts as a compensation resulting in faster growth and an increased investment in reproductive tissues.

According to their response to herbivore activities, plant species can be classified into grazing increaser and grazing decreaser species. *Helianthemum nummularium* is an example of such a grazing increaser species as this normally rare species remarkably increased its distribution shortly after the introduction of large herbivores in several grassland areas. Nevertheless, its seeds lack any obvious dispersal-related adaptations, and direct observations of plant or seed consumption are scarce. In a case-study, the dispersal suitability of *H. nummularium* via endozoochory and epizoochory was examined through field and laboratory experiments (**chapter 6**). In a differentiated grazer exclusion experiment, evidence was found that plants are grazed by both large domestic ungulates (cattle or horses) and small wild herbivores (rabbits or hares). Direct evidence of endozoochory, however, remains scarce. No seeds were found germinating in field-collected dung, and only a few seedlings emerged following a seed feeding experiment. However, once deposited, higher growth rates were found when seeds were mixed with dung, while on the other hand establishment success lowered when seeds were grown in combination with competitively superior species such as *Trifolium pratense*. Epizoochory evidence is stronger: this dispersal mechanism is plausible since both the fur and hooves of cattle and horses were found to potentially contribute to the

transport of *H. nummularium* seeds although this was hardly the case in high and dense vegetation. The increasing cover of *H. nummularium* after introducing domestic herbivores is most probably the result of a complex interplay between various ecological processes. Herbivores certainly have played a role in the dispersal of *H. nummularium* seeds through internal and external ways while their selective grazing behaviour may have created an appropriate environment for *Helianthemum* establishment and maintenance.

Primary dispersal by animals may thus evoke changes in the composition of plant communities but it may also cause considerable seed losses. Many seeds do not survive the destructive environment of the digestive tract. When seeds manage to survive the mechanical and chemical digestive processes, they often need more time to germinate and the establishment of seedlings is constrained by the dung environment. Nevertheless, the initially inhibiting dung environment becomes useful once seedlings are established and the dung starts to decompose. Herbivore dung may, therefore, provide a nutritive substrate which affects the later developmental stages of growth and flowering. Although plants initially pay a very high price for endozoochorous transport, they may recover a part of these costs later. As a consequence, the post-dispersal fate of seeds is closely linked with the fate of dung, and its decomposition rate. Although dung decomposition is affected by many factors, such as humidity, temperature and dung decomposing organisms, the activity of dung beetles is particularly interesting regarding endozoochory. Dung beetles are excellent secondary seed dispersers as they move considerable amounts of dung and accidentally bury the endozoochorous seeds present in dung particles. Related with their dung removing and burying behaviour, dung beetles fulfil several key functions in terrestrial ecosystems throughout the world. These include nutrient cycling, soil bioturbation, plant growth and parasite control. In a field experiment at two sites in the coastal dune and Campine ecoregion in Belgium, regional differences in dung beetle assemblage composition were related with dung removal and secondary seed dispersal (**chapter 7**). Dung beetles were classified in three functional groups, depending on their size and dung manipulation method: dwellers, large and small tunnelers. Other soil inhabiting fauna such as earthworms were included as a fourth functional group. Dung removal and seed dispersal by each individual functional group and combinations thereof were

estimated in exclusion experiments using different dung types. Dwellers were the most diverse and abundant group, but tunnelers were dominant in terms of biomass. All dung beetle functional groups had a clear preference for fresh dung. The ecosystem functions of dung removal and secondary seed dispersal provided by dung beetles were significant and differed between functional groups. Although in absolute numbers more dwellers were found, large tunnelers were disproportionally important for dung burial and seed removal. In the absence of dung beetles, other soil inhabiting fauna, such as earthworms, partly took over the dung decomposing role of dung beetles while most dung was processed when all native functional groups were present.

In **chapter 8**, a similar experiment was conducted at a larger, continental scale. In a multi-site experiment at 17 study sites in different biogeographic and climate zones in the Western Palaearctic, the dung and seed removing activities of dung beetle assemblages with manipulated functional compositions were examined. In this experiment, dung beetle species were classified into five functional groups according to nesting behaviour and size: dwellers, small and large tunnelers, and small and large rollers. By constructing different exclosure types that allowed or restricted the dung and seed removing activities of certain groups of the local dung beetle assemblage, we estimated the role of each functional group in the ecosystem functions of dung removal and secondary seed dispersal. Dung beetle assemblages clearly differed along a north-south and east-west gradient, with higher species and functional group richness and dung beetle abundance at lower latitudes. Northernmost sites were dominated by dwellers while a functional shift towards more tunnelers was found in the southern sites. Small rollers were found in some regions albeit with very low abundance and species richness, while large rollers were not found at any of the study sites. The higher species richness and dung beetle abundance in the southern sites resulted in higher dung removal rates. Tunnelers and rollers were more effective dung removers than dwellers, and other soil macro-invertebrates partially took over the dung removing activities of tunnelers in the dweller dominated northern sites. A positive interaction between functional group richness and dung removal rates was found and consequently stresses the need of functional group conservation in order to maintain the ecosystem functions of dung removal and the hereto linked secondary seed dispersal.

12 Samenvatting

De bewegingsopties van zittende organismen zijn eerder beperkt. In tegenstelling tot de meeste dieren die zich actief kunnen verplaatsen, zijn planten hoofdzakelijk beperkt tot passieve verbreidingsprocessen door middel van speciale structuren. De meest voorkomende structuren voor de verbreiding van planten zijn zaden of vruchten, hoewel sommige plantensoorten gespecialiseerde vegetatieve structuren hebben voor dispersie (zoals wortelstokken, bulbils of zelfs de gehele plant). Omdat de productie van een zaad de laatste stap betekent van de voortplantingscyclus van de moederplant en zaadkieming het begin van nieuw leven is, kan de verbreiding van zaden van de ene plaats naar de andere bovendien ook beschouwd worden als een mobiele link tussen generaties. Ondanks de beperkingen die planten ondervinden door het gebruik van passieve verbreiding bestaat er een grote variatie in methoden en kunnen wind, water, zwaartekracht en dieren als vector dienen. Voor natuurbeheersdoeleinden worden er vaak kuddes grote grazers zoals runderen, schapen en paarden geïntroduceerd in de halfnatuurlijke graslanden van West-Europa. Alhoewel deze hoefdieren voornamelijk ingezet worden om ruimtelijke heterogeniteit te creëren en de opslag van houtige planten tegen te gaan, beïnvloeden deze grazers de vegetatiestructuur en -samenstelling ook door plantenzaden te verspreiden.

Het is gekend dat grote grazers in gematigde graslanden gigantische hoeveelheden zaden kunnen verspreiden van een breed spectrum aan soorten. Herbivoren kunnen zaden zowel inwendig als uitwendig transporteren. In het geval van *endozoöchorie* worden er zaden tijdens het grazen ingeslikt waarna ze doorheen het spijsverteringsstelsel reizen om zo uiteindelijk in mest afgezet te worden. Voorts kunnen zaden of vruchten ook tijdelijk aan de vacht of hoeven blijven hangen om zo gebruik te maken van het bewegende dier (*epizoöchorie*). Endozoöchorie is een bijzonder complexe dispersiemethode en omvat de opname van zaden, de beweging van de zaden in het spijsverteringsstelsel, de excretie met mest, en alle daarop volgende processen met betrekking op kieming, vestiging, groei en maturatie in de nieuwe omgeving. In de afgelopen decennia is er afdoende bewijs verzameld voor het bestaan en de relevantie van endozoöchore verbreiding in halfnatuurlijke graslanden. De meeste van deze studies

gingen de zaadinhoud van op het terrein ingezamelde mest na door middel van kiemexperimenten. Als dusdanig geven deze studies een bijzonder waardevol inzicht in de verbreidingskans van veel graslandsoorten, maar weinige studies hebben ook de gevolgen en de kwalitatieve aspecten van endozoöchore verbreiding onderzocht. Niettemin kunnen de specifieke kenmerken van de herbivoor en de kwaliteit van de nieuwe omgeving waarin het zaad terecht komt een beslissende invloed hebben op het uiteindelijke lot van het zaad.

In **hoofdstuk 2** werden de verschillende aspecten van het spijsverteringsstelsel van herbivoren bestudeerd. De kieming van graslandplanten werd getest in een gestandardiseerde testomgeving na behandelingen die welbepaalde aspecten van de spijsvertering simuleerden. Runderen (herkauwers) paarden (hindgut fermenters) werden als modelorganismen gebruikt voor de experimenten. Drie belangrijke verteringsprocessen werden bestudeerd door middel van mechanische, thermische en chemische behandeling van de zaden die respectievelijk het kauwen, de lichaamstemperatuur en spijsverteringssappen in de maag nabootsten. Een selectie van 15 relatief algemene graslandsoorten werd gemaakt en binnen eenzelfde geslacht werden indien mogelijk éénjarige en meerjarige soorten uitgezocht. De gekozen soorten behoorden tot families van de Cistaceae, Cyperaceae, Fabaceae, Poaceae en Urticaceae. De gesimuleerde vertering door runderen en paarden had een vergelijkbaar effect op de kieming, maar grote verschillen in kiemgedrag werden gevonden tussen plantensoorten onderling. Voor de meeste van de geteste plantensoorten, hadden de behandelingen een remmende werking op de kieming in vergelijking met onbehandelde zaden. Echter, soorten van de Cistaceae en Fabaceae ondervonden een positief effect van de mechanische behandelingen. De thermische behandelingen die de lichaamstemperatuur simuleerden hadden in de meeste gevallen een sterk inhiberend effect op de kieming. Hoewel bepaalde soorten onvermijdelijk een hoge tol betaalden voor endozoöchore verbreiding door het verlies van kiemkrachtige zaden, werden soorten van de Cyperaceae en Poaceae nauwelijks aangetast door de behandelingen en sommigen kiemden zelfs beter na chemische behandelingen. Bovendien was het kiemingssucces van *Urtica urens* zelfs hoger na elk van de behandelingen, wat suggereert dat de zaden van deze soort bijzonder goed zijn aangepast aan de maag-darm passage, en derhalve aan endozoöchore dispersie.

Door de verschillende opname van zaden en het verlies van zaad van bepaalde soorten tijdens de spijsvertering, fungeren hoefdieren als een soort ecologische filter in de eerste stap van de cyclus van endozoöchore verbreiding. In de volgende stappen van kieming, vestiging en groei, bepalen omgevingsfactoren het lot van de gedefaeceerde zaden. De chemische samenstelling en de specifieke structuur van de mest fungeert mogelijk als een tweede filter voor endozoöchoor verspreide zaden. In **hoofdstuk 3** werden de kans op kieming en de timing hiervan experimenteel getest in de mest van runderen en paarden voor 15 eenjarige en meerjarige gematigde graslandsoorten. De kiemkracht en het ogenblik van kieming verschilde grotendeels tussen de soorten, maar in alle geteste soorten leidde de aanwezigheid van mest tot een veel latere kieming en een verlaagde kiemkracht. Deze negatieve effecten waren sterker als er rundermest werd gebruikt, wat verklaard kan worden door de verschillende structurele samenstelling. Hoewel paardenmest veel compacter was dan rundermest op het moment van afzetting, verscheen er snel een harde korst op rundermest. Bovendien is de hogere concentratie aan nutriënten in rundermest irrelevant in de kiemingsfase omdat jonge zaailingen grotendeels teren op de reserves van mineralen en organische voedingsstoffen in de zaadlobben, en is het grootste deel van deze voedingsstoffen niet direct opneembaar voor planten. Het lijkt er dus op dat de vezelachtige en lossere structuur van paardenmest minder beperkend was voor de kieming van zaden en dat vooral de harde korst op rundermest de penetratie van jonge zaailingen belemmerde.

Na de kiemingsfase echter, kunnen zaailingen in toenemende mate profiteren van de voedingsstoffen die vrijkomen uit de ontbindende mesthoop. In een poging om het effect van de mestomgeving op de latere stadia van de plantenontwikkeling te kwantificeren werden de biomassa en groeisnelheid van zaailingen, en de hoogte, vertakkingsgraad, bloei en biomassa van uitgegroeide planten gemeten (**hoofdstuk 4**). In de vroege ontwikkelingsfase, heeft de mest nauwelijks een impact op de biomassa van zaailingen. In de latere groeifasen daarentegen ondervonden de meeste soorten voordelen van de aanwezige mest, die zich uitten in een snellere groei, meer bloemen en een hogere biomassa van volwassen planten. Bijgevolg worden het initiële verlies van levensvatbare zaden in het spijsverteringssysteem van herbivoren en de verlaagde kieming in mest

gedeeltelijk gecompenseerd door een verhoogde groei en bloei in sommige soorten.

Afhankelijk van de soort herbivoren en seizoenale omstandigheden kunnen mesthopen grote hoeveelheden zaden van een groot aantal soorten bevatten. Doordat de mesthoop slechts een beperkte grootte heeft, kan er hierdoor een sterke competitie voor ruimte, licht en voedingsstoffen ontstaan tussen individuen van dezelfde soort en andere soorten. In een omgeving die rijk is aan voedingsstoffen, zoals dat het geval is met herbivore mest, kan de uitkomst van inter- en intraspecifieke competitie verschillen van situaties waarin voedingsstoffen beperkt zijn. Bovendien kan het tijdelijke eilandkarakter van mest de competitieve interacties beïnvloeden door de verschillende concentraties en soortencombinaties van endozoöchoor verspreide zaden. Er kan daarom verwacht worden dat er complexe interacties bestaan tussen verschillende zaaddichtheden, soortencombinaties en de kwaliteit en structuur van de mest. In **hoofdstuk 5** werden de verschillende aspecten van competitie tussen drie algemene meerjarige graslandsoorten onderzocht in een serre-experiment. *Agrostis stolonifera*, *Trifolium pratense* en *Trifolium repens* werden enerzijds gebruikt in combinaties met twee soorten en met verschillende verhoudingen van elke soort en anderzijds in monoculturen. Drie verschillende zaaddichtheden werden getest en de impact van runder- en paardenmest op de vestiging, groei en bloei werd gemeten. De interacties verschilden tussen de soortenmengsels en werden gerelateerd aan interspecifieke competitie. Zaden in meersoortige combinaties kiemden over het algemeen sneller, maar de resulterende zaailingen groeiden trager. De aanwezigheid van concurrerende soorten leidde tot een toename van de biomassa van soorten in polyculturen terwijl intraspecifieke concurrentie werd gevonden in monoculturen. Er werden relatief meer bloemen geteld wanneer de planten groeiden in polyculturen. Hoewel er maar weinig directe effecten van de zaaidichtheid gevonden zijn, leidden hogere dichtheden tot een lager vestigingssucces. Zoals in de voorgaande experimenten had de toevoeging van mest een negatief effect op de tijd die nodig was om tot kieming te komen en werd er een versnelde groei gemeten. Paadenmest gaf in vergelijking met rundermest aanleiding tot een sterkere interspecifieke competitie die gemeten werd door een lager vestigingssucces van zaailingen, een lagere biomassa en meer bloemen. Het lot van zaden na de initiële verbreiding wordt met andere woorden bezegeld door de dichtheid

van zaden in de mesthoop en de specifieke aanwezigheid van mest. Terwijl hoge zaaddichtheden een kost met zich meebrengen door het verlies zaad, wordt een deel van die kost gecompenseerd door een snellere groei en een toegenomen investering in reproductieve weefsels.

Naargelang hun reactie op begrazing, kunnen plantensoorten worden ingedeeld in toenemende (*grazing increaser*) en afnemende (*grazing decreaser*) soorten onder begrazing. Het Geel zonneroosje (*Helianthemum nummularium*) is een goed voorbeeld van een toenemende soort omdat de distributie van deze zeldzame plantensoort kort na de introductie van grote grazers in kustgraslanden opmerkelijk toenam. Desalniettemin hebben de zaden geen voor de hand liggende aanpassingen die naar de één of andere dispersiemethode wijzen. Voorts zijn directe waarnemingen van begrazing van het Geel zonneroosje erg schaars. In een case-studie werden de opties voor endozoöchore en epizoöchore verbreiding van het Geel zonneroosje tegen elkaar afgewogen aan de hand van veld- en labo-experimenten (**hoofdstuk 6**). Door de plant af te schermen van welbepaalde herbivoorsoorten werd bewijs gevonden van de begrazing door grote hoefdieren (paarden of koeien) en kleine wilde herbivoren (konijnen of hazen). Directe bewijzen voor endozoöchorie waren echter schaars omdat er geen zaden kiemden in de op het terrein ingezamelde mest en omdat er slechts weinig zaden ontkiemden na een voederexperiment. Er werd een snellere groei vastgesteld wanneer zaden in mest ontkiemden, terwijl er minder zaailingen in staat waren uit te groeien indien ze samen in één pot met competitief sterkere soorten zoals *Trifolium pratense* gezaaid werden. Het bewijs voor epizoöchorie is echter sterker want zowel de vacht als de hoeven van runderen en paarden bleken geschikt te zijn om zaden te vervoeren al was dit nauwelijks het geval in hoge en dichte vegetatie. De gestegen bedekking van het Geel zonneroosje is dus hoogstwaarschijnlijk te wijten aan een complexe wisselwerking tussen verschillende ecologische processen. Begrazing kan zeker een rol spelen in de verbreiding van zaden (zowel epi- als endozoöchoor) terwijl het selectieve graasgedrag van paarden en runderen een geschikte omgeving voor de kieming en vestiging van het Geel zonneroosje kan hebben gecreëerd.

Primaire dispersie door dieren kan dus leiden tot veranderingen in de samenstelling van plantengemeenschappen, maar het kan een aanzienlijk verlies van zaden veroorzaken. Veel zaden overleven de destructieve omgeving van het spijsverteringskanaal niet. Wanneer zaden erin slagen om

de levensvatbaar te blijven na de mechanische en chemische spijsverteringsprocessen, hebben ze vaak meer tijd nodig om te ontkiemen, terwijl de vestiging van zaailingen beperkt wordt door de harde structuur van mest. In een latere fase kan de aanwezigheid van mest positieve effecten hebben op de groei van planten eens de organische verbindingen beginnen af te breken tot voor de plant opneembare minerale verbindingen. Herbivore mest kan derhalve beschouwd worden als voedingssupplement dat de latere ontwikkelingsstadia van groei en bloei beïnvloedt. Hoewel planten in eerste instantie een zeer hoge prijs betalen voor endozoöchorie, kunnen ze een deel van deze kosten later recupereren. Als gevolg daarvan is het lot van zaden na endozoöchorie nauw verbonden met het lot van de mest, en de snelheid waarmee mest afgebroken wordt. Hoewel de decompositie van mest wordt beïnvloed door vele factoren, zoals het vochtgehalte, de temperatuur en de aanwezigheid van mestafbrekende organismen, is de activiteit van mestkevers bijzonder interessant met betrekking tot endozoöchorie. Mestkevers kunnen namelijk de afgezette zaden een tweede keer verplaatsen door de mest waarin zaden ingebed zitten te verplaatsen (*secundaire dispersie*). Het is gekend dat mestkevers vaak grote hoeveelheden mest verplaatsen en zich niets aantrekken van de zaden die in de mest zitten zolang de zaden niet te groot zijn in vergelijking met de kever. De meeste taken die mestkevers wereldwijd vervullen in terrestrische ecosystemen hebben te maken het verwijderen en begraven van mest, zoals de afbraak van meststoffen, bodemverluchting, het bevorderen van plantengroei en de bestrijding van parasieten. In een veldexperiment op twee locaties in de kustduinen en de Kempische ecoregio in België, werden regionale verschillen in de samenstelling van mestkevergemeenschappen verband gebracht met mestverwijdering en secundaire zaadverbreiding (**hoofdstuk 7**). Mestkevers werden ingedeeld in drie functionele groepen, afhankelijk van hun grootte en de manier waarop ze met mest omgaan: dwellers, grote en kleine tunnelers. Andere bodembewonende fauna zoals regenwormen werden opgenomen als een vierde functionele groep. De verwijdering van mest en zaden door elke functionele groep afzonderlijk en combinaties hiervan werd ingeschat door bepaalde groepen de toegang tot een experimentele mesthoop te ontzeggen. Dwellers waren de meest soortenrijke en abundante groep, maar tunnelers hadden de hoogste biomassa. Alle functionele groepen hadden een duidelijke voorkeur voor verse mest. Mestkevers verwijderden siginificante hoeveelheden mest en speelden een belangrijke rol in de

secundaire verbreiding van zaden, maar de grootte-orde verschilde tussen de functionele groepen. Hoewel er in absolute aantallen meer dwellers werden gevonden, waren grote tunnelers disproportioneel belangrijk voor de verwijdering van mest en zaden. Bij het ontbreken van mestkevers namen andere bodembewonende fauna, zoals regenwormen, deels de rol van mestkevers over terwijl de meeste mest verwijderd werd indien alle inheemse functionele groepen aanwezig waren.

In **hoofdstuk 8** werd een soortgelijk experiment uitgevoerd op een grotere, continentale schaal. In een multi-site experiment in 17 studiegebieden in verschillende biogeografische en klimaatzones in de Westpalearctische zone, werd de rol van de verschillende functionele groepen in de verwijdering van mest en zaden onderzocht door de samenstelling van mestkevergemeenschappen artificieel te manipuleren. In dit experiment werden mestkeversoorten ingedeeld in vijf functionele groepen volgens hun nestgedrag en grootte: dwellers, kleine en grote tunnelers, en kleine en grote rollers. Er werden verschillende types kooitjes gemaakt die de mest- en zaadverwijderende activiteit van bepaalde functionele groepen al dan niet toelieten. Hiermee werd vervolgens geschat welke rol elke functionele groep speelt in de verwijdering van mest en zaden. Mestkevergemeenschappen verschilden duidelijk volgens een noord-zuid en oost-west gradiënt, met meer soorten, functionele groepen en individuen op lagere breedtegraden. De meest noordelijke locaties werden gedomineerd door dwellers, terwijl een functionele verschuiving naar meer tunnelers werd gevonden in de zuidelijke sites. Kleine rollers werden gevonden in sommige regio's, zij het in zeer lage aantallen en met weinig soortenrijkdom. Er werden echter geen grote rollers gevangen. De hogere soortenrijkdom en mestkeverdensiteit in de zuidelijke gebieden resulteerde in meer mestverwijdering. Tunnelers en rollers speelden een meer prominente rol in de verwijdering van mest dan dwellers. Andere bodembewonende macro-invertebraten namen gedeeltelijk de mestverwijderende activiteiten van tunnelers over in de door dwellers gedomineerde noordelijke studiegebieden. Een positieve wisselwerking tussen de diversiteit van functionele groepen en mestverwijdering werd gevonden wat wijst op de noodzaak van het behoud van alle functionele groepen om ecosysteemfuncties zoals de verwijdering van mest en secundaire zaadverbreiding te behouden.

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Endozoochory, or the dispersal of plant seeds via ingestion by vertebrate animals and the consecutive deposition of seeds in dung, is a widespread phenomenon in temperate grassland species. The first steps in this dispersal cycle have been extensively studied in recent decades while less is known about the next steps, or the post-dispersal fate of deposited seeds. In this PhD the germination, establishment, growth and flowering of temperate grassland species was experimentally assessed through dung addition and inter- and intraspecific competition experiments. Furthermore, the role of different functional groups of dung beetles in secondary seed dispersal and dung degradation has been studied in a large-scaled multi-site experiment in different climate and biogeographic zones in the Western Palaearctic.

Keywords: dung beetles, dung removal, dispersal success, endozoochory, functional diversity, germination, plant competition, secondary seed dispersal, seed dispersal